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**14. ABSTRACT**
The nonantimicrobial Chemically Modified Tetracyclines (CMTs) inhibit invasiveness and metastasis in tumors. This study examined CMTs as inhibitors of the angiogenic response in human breast cancer cell lines, a tumor-infiltrating macrophage model and endothelial cells. CMT 308, a 9-amino derivative of CMT 300 (6-demethyl-6-deoxy-4-dedimethyl-aminotetracycline) reduced basal VEGF secretion from MCF-7 and MDA-MB-453 cells (early breast cancer models) and from MDA-MB-435s and MDA-MB-231 cells (late stage disease models). Sub-cytotoxic doses of CMT 308 also specifically reduced basal VEGF secretion from MonoMac 6 cells (a model of tumor-infiltrating macrophages). In time course studies with MCF-7 cells, VEGF secretion was detected within 4 hours of plating but addition of CMT 308 inhibited further VEGF secretion for up 8 hours. In MonoMac 6 cells, CMT 308 suppressed VEGF secretion for at least 24 hours. CMT 308 inhibited new secretion of VEGF but did not affect pre-existing VEGF levels. Treatment of breast tumor cells with TGFβ increased intracellular and secreted VEGF protein levels, but not VEGF mRNA levels. CMT 308 decreased levels of TGFβ-induced VEGF protein in MDA-MB-435s cell lysates. CMT 300, like CMT 308, did not affect VEGF mRNA levels in the breast cancer cells, but unlike CMT 308, CMT 300 did not diminish levels of secreted VEGF protein. In contrast to its effects on breast tumor lines, CMT 308 decreased VEGF mRNA levels as well as secreted VEGF protein levels in MonoMac 6 cells. Both CMT 300 and CMT 308 reduced tube formation, migration and invasion of endothelial cells through layers of Matrigel in the absence of toxicity. These results indicate that CMT 308 acts at a post-transcriptional level to inhibit basal secretion, as well as some fraction of TGFβ-stimulated secretion of VEGF in breast cancer and monocytic cells. The capacity of CMT 308 to inhibit release of VEGF as well as the angiogenic response of endothelial cells, offers promise for its development for treatment of breast cancer.

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## Table of Contents


**Cover** 1

**SF 298** 2

**Table of Contents** 3

**Introduction** 4

**Body** 4-17

- **Tasks 1 and 2** ***Effects of CMTs on VEGF Release by Breast Tumor Lines in the Absence of Growth Factors; Effects of Growth Factors on Release of VEGF by Breast Tumor Cell Lines in the Absence and Presence of CMTs*** 5-11
- **Task 3** ***Effects of CMTs on Release of VEGF by Mono Mac 6 Cells*** 11-14
- **Task 4** ***Effects of CMTs on Human Endothelial Cells*** 14-17
- **Tasks 5 and 6** ***Effects of Tumor Cell- and Macrophage-Derived Angiogenic Factors on Endothelial Cells and Inhibition of Endothelial Responses by CMTs*** 17

**Key Research Accomplishments** 17-20

**Reportable Outcomes** 20

**Conclusions and Implications** 20-30

**References** 31-35

**Table** 36

**Legends** 37-44

**Figures** 45-82

**Appendices (Era of Hope abstracts for 2002 and 2004, manuscript accepted for publication in *Cytokine*, list of participating personnel)**

## A. Introduction

The long term objective of this project has been to evaluate the capacity of a group of nonantimicrobial chemically modified tetracyclines (CMTs) to downregulate the angiogenic response which maintains breast tumor growth and proliferation. Our strategy has been based on two potential modes of action of these agents. Breast tumor angiogenesis *in vivo* is correlated with the capacity of the tumor cells as well as infiltrating macrophages to release soluble pro-angiogenic factors, including Vascular Endothelial Growth Factor (VEGF), which induce endothelial cell proliferation and differentiation into structures which assume the characteristics of neovessels. Based on observations of *in vivo* antiangiogenic activity of 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-300) in patients with Kaposi's sarcoma, we have hypothesized that the CMTs could downregulate VEGF production, could inhibit endothelial cell neovessel formation, or could act by a combination of these two modes of action. Clinical trials of CMT-300 in management of Kaposi's sarcoma have progressed to Phase II studies, and continue to offer promising results [1]. Reports on antivasculogenic and antiangiogenic effects of the CMTs *in vitro* offer further support for the potential application of these agents as inhibitors of breast tumor angiogenesis [2,3]. While CMT-300 continues to show a high level of safety, not only in NCI-sponsored clinical trials on refractory cancer patients but also in Phase I trials sponsored by the manufacturer. Collagenix Pharmaceuticals, Inc., in normal human volunteers and in patients with acne and rosacea, we recognize that this agent resembles tetracycline antibiotics in its capacity to produce phototoxicity in some patients who are exposed to sunlight without sunscreen protection [4]. In contrast, the 9-amino derivative of CMT-300 (CMT-308) shows minimal phototoxicity *in vitro* and in animal models, and has been evaluated in this project in parallel with its parent compound for its capacity to inhibit release of VEGF and to block neovessel formation by endothelial cells. In this final report we summarize our further progress in characterizing the effects of these two CMTs on release of pro-angiogenic factors by a total of four human breast tumor cell lines and a monocytoid line; on the capacity of human endothelial cells to undergo a shape change analogous to the formation of so-called "tubes" when plated on Matrigel or type I collagen; and on migration and invasion of endothelial cells towards VEGF or tumor cell conditioned media as chemoattractants.

## B. Body

This project focused on six Tasks which we established for the duration of support: to evaluate CMTs for effects on angiogenic factors released by breast tumor cell lines maintained under "basal" growth conditions (Task 1); to evaluate modulation of angiogenic factor release from the tumor cell lines by hormones, cytokines, and growth factors and to determine effects of the CMTs on these modulated levels of angiogenic factor release (Task 2); to evaluate effects of the CMTs on release of angiogenic factors by macrophages and a human macrophage cell line, Mono Mac 6 (Task 3); to evaluate capacity of the CMTs to inhibit tube formation and invasiveness of endothelial cells in response to pro-angiogenic factors (Task 4); to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to breast tumor cell lines in a co-culture system (Task 5); and to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to monocytoid cells in a co-culture system (Task 6).

***1. Task 1 - Effects of CMTs on VEGF Release by Breast Tumor Lines in the Absence of Growth Factors***

***Task 2 - Effects of Growth Factors on VEGF Release by Breast Tumor Cell Lines in the Absence and Presence of CMTs***

**a. Identifying Tumor Cell Lines That Serve as Models of Different Metastatic Stages and Angiogenic Potential in Breast Cancer**

Tumors from different stages of breast cancer present with different profiles of markers. To determine whether different cell lines that have been proposed as models of different stages of the cancer would secrete varying levels of angiogenic factors, VEGF levels were examined in four different breast tumor cell lines. MCF-7 and MDA-453 are estrogen responsive breast cancer cell lines that have low metastatic potential, and are therefore considered models of early breast cancer. Two other cell lines, MDA-MB-231 and MDA-MB-435s are estrogen receptor-negative, have high metastatic potential and are models for advanced breast cancer [5]. To establish whether the baseline levels of VEGF secreted by these cell lines would reflect their metastatic potential, VEGF secretion was examined in all four cell lines. Our results show that all four cell lines secrete basal levels of VEGF into the conditioned media. The two highly invasive cell lines (MDA-MB-231 and MDA-MB-435s) secrete higher levels of VEGF than the two weakly invasive cell lines (MDA-MB-453 and MCF-7). Of the two weakly invasive cell lines, MCF-7 cells secreted higher levels of VEGF, and of the two strongly invasive cell lines, MDA-MB-435s cells secreted higher basal levels of VEGF protein. MDA-MB-435s cells produce 8-fold more endogenous VEGF than MCF-7 cells (Table 1). Our results show that in breast cancer cell lines, basal levels of endogenous VEGF production can be correlated to the metastatic and invasive phenotype of the cell line.

**b. TGFβ Specifically Stimulates VEGF Secretion from Weakly and Aggressively Invasive Breast Cancer Cell Lines**

In addition to producing basal levels of endogenous VEGF, many tumors have the capacity to increase VEGF production upon stimulation with growth factors. TGFβ levels are increased in the serum of patients with breast cancer [11] and high TGFβ levels are correlated with high VEGF levels in breast cancer patients [12]. In certain cell types such as osteoblasts, cultured keratinocytes [13] lung carcinoma [14] cells and fibroblasts [15]. TGFβ has been shown to induce VEGF production. The ability of TGFβ to modulate VEGF production by breast cancer cells was examined. When the four cell lines were treated for 24h with increasing concentrations of TGFβ (0.1, 1.0 & 10 ng/ml), a corresponding dose-dependent increase in VEGF secretion by these cells was consistently observed. At the highest concentration of TGFβ employed (10 ng/ml), VEGF levels secreted by MCF-7 cells were increased by 57%; VEGF secretion from MDA-MB-453 cells was increased by 291%, from MDA-MB-231 cells by 251% and from MDA-MB-435s cells by 403% (Fig 2). Thus, VEGF secretion from the weakly invasive cell lines was stimulated to a lesser extent by TGFβ than from the more invasive cell lines. This confirmed the observation made by Donovan et al [12] who also identified a positive correlation of VEGF release with TGFβ levels in MDA-MB-231 cells. Contrary to its effect on stimulation of VEGF secretion, TGFβ did not induce secretion of bFGF in the MCF-7 cells, and MCF-7 cells did not secrete significant basal levels of bFGF in the absence of growth factors (Fig 3).

Another growth factor implicated in angiogenesis is IGF-I (Insulin-like Growth Factor-I) [16] IGF-I is known to induce VEGF in colon cancer cells [17] and endometrial carcinoma cells

[18]. Although IGF-I is known to induce VEGF in these other cell types, we did not see a significant response to IGF-I in the poorly invasive MCF-7 cell line (Fig 4A). Although IGF-I did stimulate significant levels of VEGF secretion in the more aggressively invasive MDA-MB-231 cell line compared to mock-treated cells, the effect of TGFβ on VEGF secretion was significantly greater (Fig 4B). This could be attributed to variations in IGF-I receptor levels in these cells. In addition, the binding protein IGFBP3 often sequesters IGF-I [19]. Perhaps the presence of increased amounts of binding protein in the conditioned media of these cells could account for the lack of response to IGF-I; however, we have not tested specifically for the complexation of IGF-I to IGFBP3 in our cultures. These results indicated that modulation of VEGF secretion is a result of cell-specific responses to growth factors, rather than a universal response to mitogens. The angiogenic factor bFGF is expressed in high levels in renal cell carcinoma tissue and in the serum of patients with leukemias and lymphomas [20]. When we assayed for bFGF secretion by the cell lines we employed, we did not find significant amounts of bFGF released by either the MCF-7 (Fig 3) or the MDA-MB-231 (data not shown) cell lines. Additionally, like TGFβ, IGF-I did not stimulate bFGF secretion in the MCF-7 cells (Fig 3). Our results are consistent with the observation that the bFGF protein lacks a signal peptide that is required for secretion [20] and secretion of bFGF into the media may require other factors.

Thus, TGFβ has been identified as a dose-dependent inducer of VEGF secretion but not of bFGF in the breast cancer cell lines that we examined, while IGF-I had no effect on VEGF secretion. Although TGFβ had no effect on the secretion of bFGF, the possibility that it induces production of bFGF, which then accumulates intracellularly, remains to be studied.

**c. Effect of CMTs on Breast Cancer Cell Lines**

In order to determine the responsiveness of tumor cells at either end of the spectrum of metastatic potential to tetracycline analogues, we employed the same four breast cancer cell lines to serve as models of various stages of angiogenic potential in breast cancer. Having established that the poorly invasive cell lines secrete lower levels of VEGF than aggressively invasive ones, we examined the effect of CMTs on the ability of these cells to secrete VEGF.

**i. CMT 308 Reduces Basal as well as TGFβ-Induced VEGF Secretion from Poorly Invasive Cell Lines**

In order to determine the effect of CMTs on VEGF secretion by poorly invasive cell lines, we evaluated two cell lines MCF-7 and MDA-453 cells. MDA 453 cells secreted slightly lower levels of basal VEGF compared to MCF-7 cells (Table 1). However, when treated with high concentrations of TGFβ, the MDA-453 cells secreted 2-fold higher levels of VEGF than correspondingly treated MCF-7 cells (Fig 2). CMT-300 produced somewhat inconsistent effects on VEGF release by MDA-MB-453 cells. When treated with 5 μM CMT 300, basal VEGF secretion in MDA-MB-453 cells was reduced by 40% in the presence of CMT 300 (Fig 5). At the higher concentration of 1 ng/ml TGFβ, however, VEGF secretion was increased by 8% in the presence of CMT 300 and at the highest concentration of TGFβ of 10 ng/ml, VEGF was decreased by 18% in the presence of CMT 300. In contrast, treatment of MDA-MB-453 cells with CMT-308 consistently resulted in diminished levels of VEGF released, regardless of the presence of TGFβ. When MDA-MB-453 cells were treated with 20 μM CMT 308, there was a 70% reduction in basal levels of VEGF secretion. CMT 308 decreased VEGF secretion from the 0.1 ng/ml TGFβ treated MDA-453 cells by 85%, and from the 1 and 10 ng/ml treated cells by 30% and 18% respectively (Fig 5).

As seen in Fig 2 and Fig 6, TGFβ stimulated VEGF secretion from MCF-7 cells. In MCF-7 cells, TGFβ at 0.1, 1 and 10 ng/ml increased VEGF secretion by 20%, 42% and 57% respectively over the levels released by untreated cells. The effects of CMTs on modulation of VEGF secretion in MCF-7 cells were also examined for comparison with the effects of these agents on MDA-MB-453 cells. CMT 300 at 5 μM did not inhibit CMT 300 at 5 μM did not inhibit basal VEGF secretion by MCF-7 cells; instead, it stimulated basal VEGF secretion by 33% at this concentration. Treatment with a higher dose of 20 μM CMT-300 resulted in even greater (130%) stimulation of VEGF secretion over basal levels (Fig 6). In contrast to the stimulation of VEGF production by CMT 300, in the presence of 20 μM CMT-308, basal levels of VEGF released by MCF-7 cells were diminished by as much as 50%. Addition of 20 μM CMT 308 also resulted in a 50% diminution of VEGF secretion in MCF-7 cells treated with 0.1 and 1 ng/ml TGFβ, and a 42% reduction in cells treated with 10 ng/ml TGFβ. Thus, TGFβ-stimulated VEGF secretion from MCF-7 cells was not diminished by concentrations of CMT 300, whereas comparable doses of CMT 308 produced significant inhibition of VEGF production.

Treatment of MCF-7 cells with IGF-I, which has been reported to contribute to pro-angiogenic activity in other systems, did not result in increased VEGF secretion (Fig 7). Additionally, CMT 308 reduced VEGF in IGF-I treated cells to the same extent as it did basal secretion (Fig 7), while CMT 300 stimulated VEGF secretion to the same extent as it did basal VEGF secretion. These experiments indicate that CMT 308 was effective in reducing levels of secreted VEGF in both poorly invasive breast cancer cell lines, whereas treatment with CMT 300 resulted in variable levels of inhibition or stimulation of VEGF release. In addition, the augmented VEGF secretion specifically induced by TGFβ is also at least partially sensitive to inhibition by CMT 308.

It has been known for some time that tetracyclines impair mitochondrial protein synthesis [21](258) Inhibition of mitochondrial protein synthesis over several cell cycles results in the arrest of cellular proliferation. Tetracyclines are therefore considered cytostatic for some cell types [21,22](258, 259). To determine whether the reduction in VEGF secretion by CMTs was a result of their cytostatic action or cytotoxicity on breast cancer cell lines, we treated MCF-7 cells with varying concentrations of either CMT 300 or CMT 308. Cytotoxicity was examined using the MTS assay, which evaluates levels of mitochondrial dehydrogenases released by viable cells. CMT 300 up to concentrations of 30 μM showed no cytotoxicity on MCF-7 cells (Fig 8). Rather, at lower concentrations from 5 μM to 20 μM CMT 300 appeared to stimulate mitochondrial activity by up to 50%, as judged by increased conversion of the tetrazolium salt to its formazan. CMT 308 displayed no cytotoxicity to MCF-7 (Fig 8) cells up to 50 μM over a 24h period. Levels of mitochondrial activity remained stable at all concentrations of CMT-308 up to the highest concentrations of CMT-300 which have been reported to be reached in the circulation of human subjects or animal models after oral administration. Thus, the reduction in VEGF secretion with CMT 308 treatment was not a result of reduced cell numbers due to cytotoxicity.

To evaluate whether inhibition of VEGF secretion from MCF-7 cells by CMT 308 occurs concurrently with the appearance of newly secreted protein, VEGF levels were measured in the conditioned medium of MCF-7 cells treated with 20 μM CMT 308 for various times. Basal VEGF production in cells that were not stimulated by a growth factor was detectable 4 hours after plating the cells in serum-free media, and rose progressively over the next 24 hours (Fig

10). In the presence of 10 ng/ml TGFβ, newly secreted VEGF could be detected in the culture medium as early as 2 hours after plating the MCF-7 cells. CMT 308 (20 μM) inhibited basal VEGF secretion at the earliest time points that the angiogenic factor could be detected in the surrounding medium of untreated control cells, and VEGF levels in the surrounding medium of CMT 308-treated cells remained undetectable up to 8 hours. Furthermore, in the medium from MCF-7 cells that were simultaneously treated with 10 ng/ml of TGFβ and 20 μM CMT 308, no VEGF could be detected up to 8h after addition of the growth factor and the tetracycline to the culture medium. During the interval between 8 and 24h after addition of the two agents, low levels of VEGF began to accumulate in the conditioned medium, indicating that the inhibitory effect of CMT 308 on VEGF was diminished after 8 hours regardless of the presence or absence of TGFβ (Fig 10).

To characterize more completely the duration of efficacy of CMT 308 in inhibiting release of VEGF, MCF-7 cells were treated with 20 μM CMT 308 either immediately after plating, or 8 hours after plating (Fig 11). Untreated cells produced significant (***$p<0.0001$) amounts of VEGF 8h and 16 h after plating. In cells that were treated at the onset with CMT 308, VEGF secretion remained undetectable over the subsequent 8h duration. In these cells CMT 308 significantly (***$p<0.0001$) reduced VEGF secretion for an interval of up to 8h after plating compared to the untreated control cells over the same duration. As the effects of CMT 308 diminished after 8h of exposure, cells produced significant amounts (***$p<0.0001$) of VEGF over the subsequent 8 hours compared to the levels of VEGF in cultures maintained during the first 8h of exposure to the tetracycline derivative. In addition, when cells were not treated with CMT 308 until 8h after plating, the accumulated VEGF levels did not change significantly over the next 8h.

Thus, the inhibitory effects of CMT 308 lasted up to 8h regardless of the time at which the cells were treated with the CMT. This confirmed the ability of the CMT to inhibit release of newly secreted VEGF, while having little or no effect on the levels of VEGF protein that had already been released into the conditioned medium. The apparent loss of efficacy of CMT-308 in inhibiting release of VEGF over time may be due to some catabolism of the CMT by the cells, or alternatively may reflect enhanced efflux of the CMT from the cell.

As seen in Fig 9, MCF-7 cells treated simultaneously with TGFβ and CMT 308 release higher levels of VEGF then those treated with CMT 308 alone for the same duration. Thus, it appears that there is at least one component of the pathway through which TGFβ enhances VEGF secretion that is not fully inhibitable by CMT 308. We further examined whether the mechanism through which TGFβ enhances VEGF secretion is at least partially sensitive to inhibition by CMT 308. We hypothesized that if CMT 308 can at least partially antagonize the augmentation of VEGF release induced by TGFβ, the levels of VEGF released in the presence of a fixed amount of TGFβ for a fixed length of time would be further modulated by the tetracycline derivative. MCF-7 cells were treated for either 16h or 24h in serum-free medium with 20 μM CMT 308, followed, without change of medium, by addition of either 10 ng/ml TGFβ or sham treatment for an additional 6h (Fig 12). Cells to which 10 ng/ml TGFβ was added for 6h after 24h in CMT 308 alone secreted higher levels (60 pg/ml) of VEGF than cells to which 10 ng/ml TGFβ was added for 6h after 16h in CMT 308 alone (23 pg/ml), although, as expected, the TGFβ-treated cells secreted more VEGF than sham treated cells over the 6h duration. If TGFβ induced a pathway for augmentation of VEGF release that was completely insensitive to CMT 308, the same amounts of VEGF would be expected to be secreted over the 6h duration of

TGFβ treatment, regardless of prior time of culture in the presence of CMT 308. Since the potency of CMT 308 to inhibit VEGF secretion gradually declines over time (Figs 9 & 10), the observation that more VEGF is secreted over 6 h in the presence of TGFβ when the growth factor is added 24 h after addition of CMT 308 than when the growth factor is added 16 h after addition of CMT 308 is consistent with a time-dependent loss of activity of CMT 308. TGFβ can stimulate MCF-7 cells to release higher levels of VEGF, but the responsiveness of the cells to the growth factor is diminished as long as some residual inhibition of VEGF release by CMT 308 persists. Thus, CMT 308 compromises the ability of cells to release increased levels of VEGF in response to a fixed amount of TGFβ for a fixed length of time.

In order to determine whether the effect of CMT-308 on diminution in levels of VEGF released by MCF-7 cells was due to reduction in VEGF mRNA levels, steady state mRNA levels in these cells lines were examined after treatment with CMTs and TGFβ using RT-PCR. Primers were chosen to distinguish between the transcripts for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ isoforms. In MCF-7 cells, the only transcripts detectable were for the secreted isoforms - $VEGF_{121}$ and $VEGF_{165}$. Neither the mRNA for the largely intracellular 189 amino acid isoform of VEGF nor the message for the 145 amino acid ECM-sequestered isoform of VEGF was detected in the MCF-7 cells.

Although dose-dependent reductions in levels of secreted VEGF protein were observed after treatment of cells with CMT 308, concentrations of this tetracycline derivative from 1 μM to 50 μM did not result in reduced VEGF mRNA levels in MCF-7 cells. (Fig 13) Additionally, TGFβ at 1 ng/ml failed to induce increased VEGF mRNA levels in MCF-7 cells although it induced augmented secretion of VEGF into the conditioned medium (Fig 13). VEGF mRNA levels in MCF-7 cells treated with 5 μM CMT 300 or 20 μM CMT 308 for 6 hours were also unaffected, regardless of pre-treatment with TGFβ (Fig 14A). When mRNA levels were measured using a quantitative assay of binding to a complementary nucleotide sequence immobilized on a multiwell microplate, the levels of VEGF message were not changed significantly after CMT 308 treatment, relative to GAPDH mRNA levels (Fig 14B). These experiments indicated that CMT 308 did not reduce the levels of VEGF protein secreted by these cell lines by modulating steady-state levels of the mRNA for VEGF.

**ii. CMT 308 Reduces Secretion of Basal As Well As TGFβ-Induced Levels of VEGF from Aggressively Invasive Cell Lines**

Since CMTs modulated VEGF secretion in the weakly invasive cell lines, we evaluated their effects on the more invasive cell lines. As see in Table 1, equivalent numbers of MDA-MB-231 and MDA-MB-435s cells secrete higher basal levels of VEGF than MCF-7 and MDA-MB-453 cells, which is consistent with their relative invasive and metastatic potential. As seen in Fig 1, in addition to this basal VEGF secretion, secretion of VEGF from the aggressively invasive tumor cells can be also augmented in the presence of TGFβ. As has been observed with the less invasive cell lines, the effects of CMT 300 on the more aggressive cell lines were variable and inconsistent. In MDA-MB-231 cells treatment with CMT 300 increased VEGF secretion in TGFβ-untreated cells by 13%, while in MDA-MB-231 cells treated with 0.1, 1 and 10 ng/ml TGFβ, CMT 300 reduced VEGF secretion by 11%, 5% and 18% respectively. Out of all the cell lines studied, CMT 300 reduced VEGF secretion only in the MDA-MB-231 cell line.

However, when treated with CMT 308 in the absence of TGFβ, MDA-MB-231 cells released 30% lower levels of VEGF, than untreated control cells, while in cells treated with 0.1, 1 and 10 ng/ml TGFβ in the presence of CMT 308, VEGF secretion was reduced by 62%, 64%

and 48% respectively when compared to levels released by cells treated with the same concentrations of growth factor in the absence of the tetracycline derivative (Fig 15).

A more pronounced effect was observed in MDA-MB-435s cells. Similar to our observations with poorly invasive MCF-7 cells, addition of neither 5 μM nor 20 μM CMT 300 could appreciably reduce VEGF secretion by MDA-MB-435s cells in the absence of TGFβ (Fig 16). TGFβ at doses of 0.1, 1 and 10 ng/ml stimulated levels of VEGF secreted by MDA-MB-435s cells by 90%, 398% and 403% respectively (Fig 16). Addition of TGFβ did not, however, alter the relative insensitivity of MDA-MB-435s cells to CMT 300: addition of this tetracycline derivative had only a minimal effect on secretion of VEGF. In contrast to the minimal efficacy of CMT 300, addition of 20 μM CMT-308 resulted in a 60% diminution in the basal levels of VEGF released by the invasive MDA-MB-435s cell line in the absence of TGFβ. Moreover, addition of 20 μM CMT-308 to cells stimulated with 0.1 and 1 ng/ml TGFβ resulted in 60% and 75% diminution of VEGF secretion respectively. At the highest concentration of TGFβ tested, 10 ng/ml,, addition of 20 μM CMT 308 resulted in a 40% reduction in VEGF secretion from the MDA-MB-435s cell line (Fig 16). Thus, two poorly invasive cell lines as well as two more aggressively invasive cell lines responded to CMT 308 treatment with a diminution in VEGF secretion.

As described above, it was observed that doses of CMT 300 up to 30 μM and doses of CMT 308 up to 50 μM were not cytotoxic to poorly invasive MCF-7 cells. To determine whether the CMTs were cytotoxic to the more aggressively invasive MDA-MB-435s and MDA-MB-231 cell lines, we treated these cells with varying concentrations of either CMT 300 or CMT 308. Cytotoxicity was examined using the MTS assay, which evaluates levels of mitochondrial dehydrogenases released by viable cells. Similar to MCF-7 cells, in MDA-MB-435s cells, doses of CMT 300 from 5 μM to 20 μM had an apparent stimulatory effect on mitochondrial dehydrogenase activity as judged by increased conversion of the tetrazolium salt to its formazan (Fig 17). Thus at low concentrations, CMT 300 appeared to induce metabolic activation of the cells. CMT 308 displayed no cytotoxicity to MDA-MB-435s (Fig 17) cells up to 50 μM over a 24h period. Levels of mitochondrial activity remained stable at all concentrations of CMT-308 up to the highest concentrations of CMT-300 that have been reported to be reached in the circulation of human subjects or animal models after oral administration. In MDA-MB-231 cells no significant toxicity was observed in the presence of doses of CMT 300 or CMT 308 up to 100 μM. Thus, the reduction in VEGF secretion with CMT 308 treatment was not a result of reduced cell numbers due to cytotoxicity.

As with the MCF-7 cells, to understand the mechanism by which culture of the MDA-MB-435s tumor line in the presence of CMT-308 resulted in diminished release of VEGF, we first determined the dose dependence of the CMT 308-mediated diminution. As noted earlier, CMT 308 inhibited VEGF secretion in MCF-7 cells in a dose-dependent manner, while CMT 300 stimulated VEGF secretion from those cells. These differing effects of the two CMTs were also observed with the more invasive MDA-MB-435s cells. Doses of CMT 300 up to 30 μM had a stimulatory effect on basal VEGF secretion, while in the presence of 50 μM CMT 300 VEGF secretion fell back to basal levels. In contrast, CMT 308 caused dose-dependent decreases in VEGF secretion from MDA-MB-435s cells in the absence of TGFβ, except at the lowest dose (1 μM) of CMT-308 tested (Fig 18). At 5 μM CMT-308, VEGF levels were diminished by 9%; this trend continued up to 50 μM at which point VEGF levels were diminished by 55%. Thus, CMT 308 diminished not only basal levels of VEGF secretion from

breast tumor lines in a dose dependent manner as seen in the MDA-MB-435s cells, but also TGFβ-augmented VEGF secretion as seen in MCF-7 cells.

In MCF-7 cells, CMTs did not modulate VEGF mRNA levels. In order to determine whether the effect of CMT-308 on diminution in levels of VEGF released by the more invasive MDA-MB-435s cells was due to reduction in VEGF mRNA levels, steady state mRNA levels in these cells lines were examined after treatment with CMTs and TGFβ using RT-PCR. Primers were chosen to distinguish between the transcripts for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ isoforms. In MDA-MB-435s cells as in MCF-7 cells, the only transcripts detectable were for the secreted isoforms of the protein - $VEGF_{121}$ and $VEGF_{165}$. Neither the mRNA for the largely intracellular 189 amino acid isoform nor the message for the 145 amino acid ECM-sequestered isoform of VEGF was detected in this cell line (Fig 19).

Notably, TGFβ at 1 ng/ml failed to induce increased VEGF mRNA levels in the MDA-MB-435s cells although it induced augmented secretion of VEGF into the conditioned medium (Fig 16). VEGF mRNA levels in MDA-MB-435s cells treated with 5 μM CMT 300 or 20 μM CMT 308 for 24 hours were unaltered from the levels in control cells (Fig 19), regardless of pre-treatment with TGFβ. The experiments indicated that CMT 308 did not reduce the levels of VEGF protein secreted by this cell line by modulating steady-state levels of the mRNA for VEGF.

To determine whether the diminished secretion of VEGF by breast tumor cell lines in the presence of CMT 308 was paralleled by alterations in the intracellular levels of VEGF, lysates of MDA-MB-435s cells treated with the CMTs were evaluated for VEGF protein by ELISA. As expected for a secreted cytokine, total steady-state intracellular VEGF levels were significantly lower (Fig 20, Left Y axis) than secreted VEGF levels which accumulated in the conditioned media (Fig 20 Right Y axis). Treatment with 1 ng/ml TGFβ significantly increased the intracellular VEGF pool similar to the augmentation of levels of secreted VEGF observed after addition of TGFβ (Fig 20). Treatment with CMT 300 also increased intracellular VEGF levels in both, the TGFβ-treated and untreated cells, reflecting the pattern observed with secreted VEGF levels. Interestingly, treatment of MDA-MB-435s cells with CMT 308 in the absence of TGFβ did not significantly reduce intracellular VEGF pools; instead, intracellular VEGF levels were similar to those in untreated cells. However, in cells treated with TGFβ, addition of CMT 308 did reduce intracellular VEGF levels analogous to the reduction seen in the secreted levels. Thus, increase in intracellular VEGF levels with TGFβ treatment occurs through CMT 308-sensitive pathways as does the secretion of the protein. The effect of CMTs on VEGF levels in cell lysates thus parallels their effect on extracellular VEGF levels.

***2. Task 3 - Effects of CMTs on VEGF Release by Mono Mac 6 Cells***

The environment of a tumor consists of the cancer cells and the surrounding stromal tissue, as well as infiltrating immune cells. These immune cells contribute to the plethora of cytokines and growth factors present in the tumor milieu. In order to evaluate the contribution of such immune cells to tumor angiogenesis, we examined VEGF levels produced by the monocytic/macrophagic cell line MonoMac 6.

**i) Cytotoxic Effects of CMTs on MonoMac 6 cells**

We evaluated the cytotoxicity of CMTs on MonoMac 6 cells using the MTS assay for mitochondrial dehydrogenase activity of viable cells. Unlike the breast cancer cell lines, which were relatively resistant to any cytotoxic effects of the chemically modified tetracyclines, the viability of Mono Mac 6 cells was diminished by 40% after 24h in the presence of 5 μM CMT-

300. In the presence of higher doses of 10 μM or 20 μM CMT 300, cell viability remained steady at 50%. We have estimated an $IC_{50}$ of 10 μM for CMT 300 in MonoMac 6 cells, although the failure to achieve complete cytotoxicity at higher doses remains unexplained. Cell viability in the presence of doses of CMT-308 as high as 20 μM over 24h was diminished by no more than 20%. The relatively low level of cytotoxicity of CMT-308 compared with that of CMT 300 allowed us to employ CMT 308 safely at higher concentrations than CMT 300 in studies with MonoMac 6 cells. Hence, CMTs 300 and 308 were used at sub-cytotoxic concentrations of 5 μM and 20 μM respectively (Fig 21).

**ii. CMT 308 Selectively Inhibits VEGF Secretion from MonoMac 6 Cells in a Dose-Dependent Manner**

Analysis of the conditioned medium from MonoMac 6 cell cultures by an ELISA for VEGF protein revealed that this monocytoid line secreted basal levels of VEGF into the surrounding medium without additional stimulation. MonoMac 6 cells were treated with 5 μM, 10 μM, and 20 μM of CMTs 300 and 308. As with the breast cancer cell lines, 5 μM CMT 300 stimulated VEGF secretion by MonoMac 6 cells by a factor of threefold. This increase was correlated with an apparent mitochondrial stimulation of MonoMac 6 cells in the presence of 5 μM CMT 300 as reflected in enhanced conversion of MTS to its formazan (Fig 22A). At 10 μM, CMT 300 appeared to have no significant effect on VEGF secretion, whereas 20 μM CMT 300 appeared to decrease VEGF secretion from MonoMac 6 cells by 56%, consistent with its cytotoxic action.

In contrast to the complex dose dependence of the effects of CMT-300 on VEGF release by MonoMac 6 cells, in the presence of increasing doses of CMT 308 from 5 μM to 20 μM, the levels of VEGF released by the cell line declined in a monotonic fashion. In the presence of 20 μM CMT-308, VEGF release was reduced to nearly undetectable levels with no significant increase in cytotoxicity. The effects of CMT 300 and CMT 308 on VEGF release by MonoMac 6 cells did not appear to reflect a more general effect of these tetracycline derivatives on protein secretion by the cell line, as total protein levels in the conditioned medium were unaffected after addition of CMT 300 or CMT 308 to MonoMac 6 cell cultures (Fig 22B).

**iii. Effects of CMT 308 in MonoMac 6 Cells are Specific for VEGF**

To examine whether inhibition of VEGF release was a reflection of a more broad spectrum inhibition of inflammatory biomarker production, the conditioned medium of MonoMac 6 cells treated with CMTs was assayed for levels of IL-8, an inflammatory cytokine which is released spontaneously at low but significant levels, using an ELISA. CMT 300 stimulated IL-8 secretion by these cells, as we had previously observed for VEGF. On the other hand, unlike its effect on VEGF release, CMT 308 did not inhibit basal levels of IL-8 secretion by these cells (Fig 23). This observation indicates that the inhibitory action of CMT 308 on VEGF secretion is not due to a general inhibition of inflammatory pathways; rather, it is limited to a subset of pathways which includes those regulating secretion of VEGF.

**iv. Kinetics of VEGF Inhibition by CMT 308 in MonoMac 6 Cells**

As seen in Fig 24, VEGF could be detected in the conditioned medium of MonoMac 6 cells within 4h of plating the cells. Subsequently, there was a time-dependent increase in VEGF protein secretion by the cells into the conditioned medium up to 24h (Fig 24) and even up to 48h (data not shown). Dose response studies (Fig 22A) showed that 20 μM CMT 308 could

dramatically reduce VEGF secretion over at least the first 24h of treatment. To establish the duration of effective inhibition of VEGF secretion in these cells, aliquots of conditioned medium were collected at various times after treatment with of MonoMac 6 cultures with 20 μM CMT 308 and were assayed for VEGF. When cells were treated with 20 μM CMT 308 at the time of plating, inhibition of secretion of VEGF was already evident at the 4 hour time point at which VEGF could be first detected in the medium of untreated cells. Nearly 100% inhibition of VEGF secretion from cells cultured in the presence of CMT 308 persisted for up to 24h (Fig 24) and further up to 48h (data not shown).

**v. TGFβ Does Not Induce VEGF Secretion by MonoMac 6 Cells**

Since TGFβ is known to induce VEGF secretion in several tumor cell lines (including the breast cancer cell lines we have studied), the effect of TGFβ on secretion of angiogenic factors by MonoMac 6 cells was evaluated. MonoMac 6 cells were treated with TGFβ as well as another pro-angiogenic growth factor, IGF-I. Concentrations from 0.1 ng/ml to 10 ng/ml of TGFβ (Fig 25) or IGF-I (Data not shown) had no effect on VEGF levels secreted by MonoMac 6 cells. In addition, basic Fibroblast Growth Factor (bFGF), an important pro-angiogenic cytokine released by some cells, was not detected in the conditioned media of MonoMac 6 cells even when stimulated with TGFβ (data not shown). A sub-cytotoxic dose of 5 μM CMT 300 did not inhibit VEGF secretion in the TGFβ-treated cells, as we had previously observed in cells cultured in the absence of any exogenous growth factor. Rather, equivalent stimulation of VEGF secretion was observed either in the absence or the presence of TGFβ, which correlated with the increase in mitochondrial activity that we also had observed in the presence of 5 μM CMT 300 (Fig 21). On the other hand, in the presence of the subcytotoxic dose of 20 μM CMT-308, VEGF levels in the conditioned media of Mono Mac 6 cells were reduced by nearly 100% regardless of the presence or absence of TGFβ.

Although TGFβ did not induce VEGF secretion, complete medium containing 10% fetal calf serum induced a significant increase in VEGF secretion from MonoMac 6 cells (Fig 26) compared to cells grown in serum-free medium. It should be noted that serum-supplemented medium itself did not contain measurable levels of VEGF (data not shown). In contrast to the inhibitory effects of CMT 308 observed in cells grown in serum-free medium, the augmented levels of VEGF released by MonoMac 6 cells in serum-supplemented medium were not reduced by treatment with CMT 308 at the sub-cytotoxic concentrations.

**vi. Effect of CMTs on Intracellular VEGF Levels in MonoMac 6 Cells**

As indicated above, MonoMac 6 cells maintained in serum-free medium secrete basal levels of VEGF into the surrounding medium. The intracellular levels of MonoMac 6 cells treated with CMTs were evaluated to determine whether the inhibitory effect of CMT 308 on VEGF release extends to modulation of intracellular pools of VEGF. Western blots of lysates of cells treated with 5 μM CMT 300 showed a reduction in the levels of the 46kD full length form of VEGF (Fig 27A). Similarly, immunoblots of lysates of cells treated with 20 μM CMT 308 showed a small decrease in the levels of an immunoreactive band at 46kD. Analysis of blots of MonoMac 6 cell lysates also revealed the presence of an immunoreactive higher molecular weight form of VEGF. Various molecular weight isoforms of VEGF have been reported by several authors [66,67] and cell-type specificity in VEGF isoform expression has also been observed in different cells. The levels of the higher molecular weight immunoreactive band detected in MonoMac 6 cell lysates were reduced somewhat after treatment of the cells with 5 μM CMT 300 and more significantly after the cells were treated with 20 μM CMT 308. The

levels of total intracellular protein in MonoMac 6 cells were not significantly altered after treatment with CMTs (Fig 27B). When conditioned medium from control MonoMac 6 cells cultured in the absence of CMTs was evaluated by SDS-PAGE and immunoblotting, the 46 kDa isoform of VEGF could be visualized. The intensity of this immunoreactive band was reduced significantly in the conditioned medium of cells maintained in the presence of CMT 300. Most dramatically, this isoform was almost completely absent in the conditioned medium of cells treated with CMT 308. In addition, no other isoforms of VEGF were observed in the conditioned media. These observations confirmed the results obtained from ELISAs of the conditioned media (Fig 25).

**vii. CMT 308 Treatment Reduces VEGF mRNA Levels in MonoMac 6 Cells**

As described above, treatment of MonoMac 6 cells with 20 μM CMT 308 resulted in a dramatic decrease in secreted levels of VEGF protein. To determine whether inhibition of VEGF secretion from MonoMac 6 cells in the presence of CMT 308 was due to inhibition at the transcriptional level, MonoMac 6 cells were cultured for 6h in the presence of the CMTs and steady state VEGF mRNA levels were examined by RT-PCR (Fig 28A). In this cell line, the only transcripts detectable were for the secreted isoforms - $VEGF_{121}$ and $VEGF_{165}$. As we had observed in the breast cancer cells we have studied, neither the mRNA for the largely intracellular 189 amino acid isoform nor the message for the 145 amino acid ECM-sequestered isoform of VEGF protein were detected in the Mono Mac 6 cell line. In cells that had either been untreated or treated with the CMTs, steady state levels of GAPDH mRNA remained unchanged. In contrast to the slight increase seen in levels of secreted VEGF protein, the levels of VEGF mRNA were slightly diminished in MonoMac 6 cells cultured in the presence of 5 μM CMT 300. Additionally, in MonoMac 6 cells cultured in the presence of 20 μM CMT 308, levels of the mRNA transcripts for both $VEGF_{121}$ and $VEGF_{165}$ were reduced slightly relative to levels of the mRNA for GAPDH. When levels of VEGF mRNA were determined quantitatively (Fig 28B), it was observed that CMT 308 treatment did result in somewhat decreased VEGF mRNA levels in MonoMac 6 cells, although this diminution was not as great as the apparently near total abrogation of VEGF protein secretion after treatment of the cells with CMT 308.

***3. Task 4 - Effects of CMTs on Human Endothelial Cells***

Endothelial cells, like tumor infiltrating inflammatory cells constitute an important component of the tumor microenvironment, by contributing to the development of new blood vessels. In previous studies, CMT-300 inhibited microtubule formation and proliferation of Human Umbilical Vein Endothelial Cells at a concentration of 10μM. In recent clinical trials, CMT-300 has been reported to halt or retard the progression of some solid tumors and to achieve either complete or significant partial regression of the angioproliferative lesions of Kaposi's sarcoma in 33% of the patients evaluated. CMTs may be more cytotoxic to proliferating cells than to the normal stromal cells. Therefore, we compared the effect of CMTs on endothelial cells cultured to ‘cobblestone’ confluence with those on proliferating, tube-forming endothelial cells that are still undergoing shape change. Examining the effect of CMTs on endothelial cells with respect to cytotoxicity, and morphological change would help identify the possible mechanisms involved in the response in patients with Kaposi's sarcoma treated with CMT 300.

**i. Cytotoxicity of CMTs on HUVEC**

We evaluated the cytotoxicity of CMTs on Human Umbilical Vein Endothelial Cells using the MTS assay for mitochondrial dehydrogenase activity of viable cells. Like the breast cancer cell lines, which were relatively resistant to any cytotoxic effects of the chemically

modified tetracyclines, the viability of HUVEC was not diminished by significantly after 24h in the presence of 10 μM CMT-300 (Fig 29). In the presence of higher doses of 100 μM CMT 300, 70% of the cells remained viable. As with the MonoMac 6 cells, the failure to achieve complete cytotoxicity at higher doses remains unexplained. Cell viability in the presence of doses of CMT-308 as high as 100 μM over 24h was not diminished significantly (Fig 29). The relatively low level of cytotoxicity of CMT-300 and CMT 308 allowed us to employ the CMTs safely at concentrations as high as 30 μM.

**ii. CMTs Inhibit Tube Formation by HMVEC Plated on Matrigel**

To evaluate whether CMTs can inhibit morphological changes leading to tube formation, HMVEC at passages 4 or 5 suspended in complete medium were treated with CMT 300 or 308 at various concentrations, and immediately plated on a thick matrigel-coated plate. The plate was incubated at $37^0$C for 18h. Cells were then washed, and stained with Calcein AM, a vital dye which is converted to a fluorescent product only in the cytosol of viable cells. After washing off excess dye, tube formation was observed under a fluorescent microscope and images recorded at 4X magnification.

We confirmed the spontaneous formation of tubes by endothelial cells when grown on a thick layer of matrigel in the absence of CMTs. Pre-treatment of HMVEC with either CMT 300 (Fig 30) or CMT 308 (Fig 31) at varying concentrations resulted in a dose-dependent inhibition of tube formation. In all cases, the tubular extensions were both qualitatively and quantitatively reduced and were completely lost at the highest doses tested, resulting in globular masses of intensely fluorescent cells. At none of these doses did calcein fluorescence disappear, indicating that loss of tube formation was not associated with cytotoxicity. In addition, cytotoxicity evaluated by the MTS assay confirmed that CMT 300 was not cytotoxic to HMVEC grown on Matrigel. HMVEC on Matrigel showed some diminution in capacity to convert MTS to its formazan at the lowest dose of CMT 308 tested, 1 μM, but at higher doses of CMT 308, there was no further loss of reductase activity (Fig 32). We have not further explored this apparent effect of CMT 308 on mitochondrial dehydrogenase activity in HMVEC.

**iii. CMTs Inhibit Tube Formation by HMVEC in Collagen I**

We had observed that CMT 300 and CMT 308 could inhibit the formation of tube-like structures by HMVEC that spontaneously form tubes on Matrigel. We then examined the ability of endothelial cells to form similar tubes when grown only on Type I Collagen. As has been shown by Whelan and Senger, endothelial cells undergo a spontaneous morphogenic transition to a tube-like reticular network when cultured in the presence of collagen I [9]. CMT 300 (data not shown) and CMT 308 (Fig 33) at concentrations of 30 μM affected morphological changes in these cells. The branches between islands of cells that were multicellular and thick in to which collagen was added in the absence of CMTs were thinner and apparently unicellular if present at all after addition of collagen in the presence of either of the CMTs. CMT 308 appeared to be slightly more effective than CMT 300 at the same concentrations in reducing the extent of the morphogenic transition. The reduction in tube structures appeared to be less dramatic than observed in cells grown on Matrigel, indicating that perhaps in the presence of a full complement of basement membrane extracellular matrix proteins endothelial cells may be more sensitive to inhibition of tube formation than in the presence of type I collagen, a component of interstitial matrix. Nevertheless the inhibition of morphological change by CMTs was significant.

**iv. CMTs Do Not Cause Morphological Change in Pre-formed Tubes of HMVEC on Collagen I**

In order to determine whether the morphogenic transition induced by addition of type I collagen to endothelial cells could be reversed by addition of CMTs, the tetracycline derivatives were added to cells in collagen which had already established reticular networks. No change was observed in these networks upon treatment with CMTs. There was no diminution in either the numbers or thickness of the branches between islands, as evaluated by phase contrast microscopy (Fig 34). Cells were treated with Calcein AM to confirm cell viability. During the process of staining, several of the branches observed in all cells with or without CMTs before staining by phase contrast were broken due to agitation. Thus, treatment of pre-formed tubes of HMVEC with CMTs did not induce morphologic changes in these tubular networks.

In order to determine whether the morphological changes could be affected after formation of the tubes, CMTs were added after tubes were formed. No change was observed in existing tubes upon treatment with CMTs. Neither the branches between islands were lost, nor were they made more unicellular as seen in the phase contrast pictures (Fig 34). Cells were treated with Calcein AM to confirm cell viability. During the process of staining, several of the branches observed before staining by phase contrast in CMT 300 and CMT 308 treated cells were broken due to agitation. Thus, treatment of pre-formed tubes of HMVEC grown on collagen I or Matrigel (data not shown) with CMTs did not induce morphologic changes in these tubular networks.

**v. CMTs Inhibit Migration of HUVEC Through Fibronectin Coated Inserts**

Since the CMTs could affect morphological changes in the endothelial cells, we examined whether migration of endothelial cells towards a chemoattractant would be affected as well. The process of migration requires morphological changes and cellular movement. We examined whether the CMTs could affect migration of HUVEC through fibronectin coated porous inserts in response to increasing concentrations of VEGF. Migration through the unoccluded pores of the fibronectin-coated membrane permits passage without the involvement of proteases such as matrix metalloproteases, which are known to be secreted by endothelial cells when they invade a basement membrane containing collagen. HUVEC showed significant dose-dependent increases in migration towards increasing concentrations of VEGF from 0.5 to 20 ng/ml compared to migration in the absence of any added chemoattractant. HUVEC migration towards 20 ng/ml VEGF was 15-fold greater than that towards medium without VEGF (Figs 35, 36). CMT 300 at 10 μM (Fig 35) appeared to have a significant inhibitory effect on HUVEC that were induced to migrate towards varying concentrations of VEGF as a chemoattractant. CMT 308 at 20 μM (Fig 36) also inhibited migration by these cells, more significantly than CMT 300, especially at higher doses of the chemoattractant VEGF. Thus it appears that CMTs may inhibit migration of HUVEC that is not dependent on proteolytic activity. This further alluded to a mechanism of action of CMT's on processes within endothelial cells that involve morphological changes rather than proteolysis.

**vi. Effect of CMTs on Invasion of HMVEC Through Matrigel Coated Inserts**

In addition to the migratory responses, the process of invasion of endothelial cells through Matrigel involves the secretion of proteases that allow passage through basement membrane components in the reconstituted matrix. In order to further understand the effects of CMTs on endothelial cell morphology, we examined their effects on the invasion of HMVEC through Matrigel-coated inserts towards the chemoattractant VEGF. The uniform Matrigel matrix layer on the inserts serves as a reconstituted basement membrane *in vitro*, providing a

barrier to non-invasive cells that are blocked from migration due to the occlusion of the membrane pores. Invasion of HMVEC towards 10 ng/ml VEGF increased 83% over invasion towards serum-free medium without VEGF (Fig 37). When HMVEC were treated with CMT 300, there was a significant decrease in invasion towards VEGF, while in the absence of VEGF an increase in invasion by CMT 300-treated cells was observed relative to control cells in the absence of chemoattractants or tetracyclines. Similarly, when treated with CMT 308, HMVEC invasion towards 10 ng/ml VEGF was significantly decreased while the invasion of CMT 308-treated cells towards media without chemoattractant was increased over mock-treated cells in the absence of chemoattractants or tetracyclines. These observations depict the ability of CMTs to inhibit chemoattractant-directed invasion of HMVEC through a basement membrane extracellular matrix.

***4. Tasks 5 and 6 - Effects of Tumor Cell- and Macrophage-Derived Angiogenic Factors on Endothelial Cells and Inhibition of Endothelial Responses by CMTs***

**i. Effect of Conditioned Media from CMT 308-Treated MonoMac 6 Cells on Invasion of HMVEC Through Matrigel Coated Inserts.**

Having demonstrated the capacity of CMTs to inhibit invasion of HMVEC towards the chemoattractant VEGF, we next evaluated the capacity of the CMTs to inhibit invasion of HMVEC towards conditioned media from MonoMac 6 cells. As described above, treatment with 20 μM CMT 308 almost completely inhibited the release of VEGF from MonoMac 6 cells, while 5 μM CMT 300 had no effect on VEGF secretion by the monocytic cell line. The undiluted conditioned media from MonoMac 6 cells treated with either of the CMTs were used as sources of chemoattractants for invasion of HMVEC through Matrigel coated inserts. Conditioned medium from MonoMac 6 cells treated with 5 μM CMT 300 significantly decreased invasion of HMVEC by 30% compared to conditioned medium from mock-treated MonoMac 6 cells. Conditioned medium from CMT 308–treated MonoMac 6 cells also induced 20% less invasion by HMVEC than conditioned medium from mock-treated cells (Fig 38). It should be noted that the conditions in these experiments resulted in exposure of the endothelial cells to the CMTs at the same concentrations that were employed for preparation of MonoMac 6-conditioned media. Thus, inhibition of endothelial cell migration in these studies might be attributed to a combination of effects of the CMTs on release of chemoattractants such as VEGF by the MonoMac 6 cells and invasive migration by the endothelial cells.

**C. Research Accomplishments - 5/15/01 - 5/15/05**

- Determined levels of VEGF released by MCF-7 (a model of early breast cancer) and MDA-MB-231 (a model of advanced breast cancer) cell lines. The MDA-MB-231 cells release higher levels of VEGF than the MCF-7 cells on a per cell basis in the absence of supplemental growth factors. Neither cell line releases detectable levels of b-FGF.
- Demonstrated inhibition of VEGF release from MCF-7 cells and MDA-MB-231 cells cultured in the presence of CMT-3 or CMT-308. At equivalent doses, CMT-308 inhibits VEGF release from both cell lines significantly more than CMT-3. Inhibition of VEGF release is detected at doses which are not cytotoxic to either cell line.
- Demonstrated augmented release of VEGF from MCF-7 cells and MDA-MB-231 cells cultured in the presence of added TGF-β; this increase in levels of VEGF is dependent upon the levels of added TGF-β.

- Demonstrated that the augmented levels of VEGF released by MCF-7 and MDA-MB-231 cell lines in the presence of TGF-β are diminished somewhat upon addition of CMT-3 and more significantly upon addition of CMT-308.
- Demonstrated that addition of IGF-1 to MCF-7 and MDA-MB-231 cell lines has no effect on VEGF release.
- Demonstrated that the human monocytic cell line Mono Mac 6 (a model of tumor infiltrating macrophages) releases VEGF in the absence of added stimuli; VEGF levels from this cell line are unaltered in the presence of TGF-β or IGF-1.
- Demonstrated that CMT-3 has minimal effect on VEGF release from Mono Mac 6 cells but CMT-308 is a very potent inhibitor of VEGF release, diminishing VEGF to virtually undetectable levels in the absence of cytotoxicity.
- Demonstrated that CMT-3 and CMT-308 are not cytotoxic to confluent human umbilical vein endothelial cells at doses which inhibit VEGF release from the two tested breast tumor cell lines.
- Characterized release of "baseline" levels of VEGF by a third human breast tumor cell line, MDA-MB-453, previously described as a model of moderately advanced breast cancer.
- Characterized dose-dependent enhancement of VEGF release from MDA-MB-453 cells by culture in the presence of TGF-β.
- Refined effects of CMT-300 and CMT-308 on release of VEGF from MCF-7 and MDA-MB-453 cells: CMT-300 at 5 μM mildly stimulates VEGF release from MCF-7 cells and modestly inhibits VEGF release from MDA-MB-453 cells, whereas CMT-308 at 20 μM inhibits release of VEGF from MCF-7 and MDA-MB-453 cells. The inhibitory effect of CMT-308 appears to be somewhat blunted by high TGF-β levels in MDA-MB-453 cells, which induce marked increases in VEGF production. A shift in the properties of the MDA-MB-231 cells previously studied in our laboratory which resulted in marked increases in VEGF production also resulted in diminished sensitivity to CMT-308.
- Established time course of release of VEGF from MCF-7 cells and suppression of VEGF release by CMT-308: demonstrated that newly released VEGF is first detected 2-4 hours after initiation of culture of MCF-7 cells in the presence or absence of TGF-β, and that 20 μM CMT-308 completely suppresses release of VEGF, also in the presence or absence of TGF-β, for up to 8 hours.
- Characterized the dose dependence of the effects of CMT-300 on VEGF release from Mono Mac 6 cells: 5 μM CMT-300 stimulates VEGF release with ~40% cytotoxicity while higher doses are so cytotoxic that net VEGF release appears to be inhibited.
- Characterized the dose-dependence of inhibition of VEGF release from Mono Mac 6 cells by CMT 308: 5 μM CMT-308 suppresses VEGF release by >50%, while 20 μM CMT-308 suppresses VEGF release by >90%, all in the absence of marked cytotoxicity.
- Characterized the time course of VEGF release from Mono Mac 6 cells and its suppression by CMT-308: demonstrated that newly released VEGF is first detected 1 hour after initiation of culture of Mono Mac 6 cells, and that 20 μM CMT-308 completely suppresses release of VEGF for up to 8 hours.
- Demonstrated that CMT-300 and CMT-308 at doses as high as 50 μM are not cytotoxic to human microvascular endothelial cells cultured on thick layers of the basement membrane matrix, Matrigel™.

- Demonstrated that CMT-300 and CMT-308 diminish tube formation from human microvascular endothelial cells cultured on thick layers of Matrigel at doses as low as 1 μM. Inhibition is apparently at least 50% at ~10 μM doses of either CMT and is complete at 30-50 μM CMT-300 or maximal and nearly complete at 30-50 μM CMT-308..
- Characterized release of "baseline" levels of VEGF by a fourth human breast tumor cell line, MDA-MB-435S, previously described as a model of highly invasive breast cancer.
- Characterized dose-dependent enhancement of VEGF release from MDA-MB-435S cells by culture in the presence of TGF-β.
- Refined effects of CMT-300 and CMT-308 on release of VEGF from MCF-7 and MDA-MB-453 cells: CMT-300 at 5 μM stimulates VEGF release from MCF-7 cells and at doses up to 20 μM also stimulates VEGF release from MDA-MB-435S cells; in both cell lines this augmented release is accompanied by increased conversion of MTS to its formazan, suggesting an "activation response." CMT-308 inhibits release of VEGF from MCF-7 and MDA-MB-435S cells in a dose dependent fashion in the absence of an "activation response" or cytotoxicity. The inhibitory effect of CMT-308 appears to be somewhat blunted by very high TGF-β levels (10 ng/ml) in MDA-MB-435S cells, but in the presence of lower TGF-β levels, CMT-308 diminishes levels of secreted VEGF by 50% or more at doses which have been demonstrated to be achieved in the blood of human patients.
- Established time course of release of VEGF from MCF-7 cells and suppression of VEGF release by CMT-308: confirmed that newly released VEGF is first detected 2-4 hours after initiation of culture of MCF-7 cells in the presence or absence of TGF-β, and that 20 μM CMT-308 completely suppresses release of VEGF, also in the presence or absence of TGF-β, for up to 8 hours. After 8 hours of exposure to CMT-308, VEGF production is no longer suppressed. If the CMT-308 is introduced after 8 hours of culture in the absence of CMTs, no additional VEGF is produced over the subsequent 8 hours, but the pre-existing VEGF levels are unaffected, indicating that the CMT inhibits release of new VEGF rather than facilitating destruction of VEGF.
- Demonstrated that augmentation of released levels of VEGF by CMT-300 and diminution of released levels of VEGF by CMT-308 in MCF-7 cells as well as MDA-MB-435S cells is not accompanied by any significant alteration in levels of intracellular VEGF isoforms or mRNA species coding for those isoforms in either cell line. This result suggests that the two CMTs affect a post-transcriptional and post-translational step, possibly at the level of secretion.
- Further characterized the dose dependence of the effects of CMT-300 on VEGF release from Mono Mac 6 cells: 5 μM CMT-300 stimulates VEGF release with ~40% cytotoxicity and while higher doses are somewhat more cytotoxic, VEGF release appears to be inhibited in a dose dependent fashion which may reflect effects on secretion.
- Confirmed the dose-dependence of inhibition of VEGF release from Mono Mac 6 cells by CMT 308: 5 μM CMT-308 suppresses VEGF release by >50%, while 20 μM CMT-308 suppresses VEGF release by >90%, all in the absence of marked cytotoxicity. The release of VEGF and its suppression by CMT-308 are unresponsive to TGF-β.
- Characterized the time course of VEGF release from Mono Mac 6 cells and its suppression by CMT-308: demonstrated that newly released VEGF is first detected ~4

hours after initiation of culture of Mono Mac 6 cells, and that 20 μM CMT-308 suppresses release of VEGF by >90% for up to 48 hours.

- Demonstrated the effects of CMTs on levels of intracellular VEGF isoforms and mRNAs for those isoforms in Mono Mac 6 cells: CMT-300 diminishes intracellular levels of the 46 kDa isoform of VEGF as well as mRNAs coding for 121 amino acid and 165 amino acid species of VEGF, while CMT-308 has only minimal effects on the levels of the intracellular levels of VEGF isoforms or the mRNA species for those isoforms.
- Demonstrated that CMT-300 and CMT-308 diminish tube-like bridge formation in human microvascular endothelial cells cultured on plastic with an overlay of type I collagen. The effects are less pronounced than those which were previously observed in endothelial cells cultured on thick layers of Matrigel, but are qualitatively similar, with significant diminution in the number of bridges and the thickness of the remaining bridges at 30 μM doses of either CMT.
- Demonstrated that CMT-300 and CMT-308 inhibit endothelial cell migration through fibronectin-coated porous membranes in response to the chemotactic stimuli of VEGF alone or VEGF+serum. Inhibition of migration by CMT-300 is more effective than by CMT-308, and is more effective when VEGF alone is employed as the chemoattractant.

**D. Reportable Outcomes**

We have presented our work on "Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer" as poster presentations at the 2002 and 2004 Department of Defense Breast Cancer Research Program Meeting "Era of Hope" meetings [10]. We have also submitted a manuscript describing the effects of CMTs on multiple breast tumor cell lines which has been accepted for publication in the journal *Cytokine*. A second manuscript describing the effects of the CMTs on Mono Mac 6 cells and on endothelial cells is now in preparation. The Era of Hope abstracts and the manuscript to be published in Cytokine are appended to the report.

**E. Conclusions**

The microenvironment of a tumor consists of various tumor-derived and stromal components including infiltrating immune cells and endothelial cells. The ultimate balance of pro- and antiangiogenic molecules released by all these tumor components determines the effective angiogenic capacity of the tumor. In this dissertation, we examine *in vitro* the ability of three components of a tumor microenvironment – the tumor cells, monocytes and endothelial cells to respond to Chemically Modified Tetracyclines, which are known anti-proteolytic anti-invasive agents. The evidence we present here that these agents also display anti-angiogenic activities targeting several components of the tumor microenvironment, makes them potentially more attractive for therapeutic applications than anti-neoplastic agents that have single-targets.

We observed that a number of breast tumor cell lines which are models of early and late stages of cancer, as well as a monocytoid line which serves as a model of infiltrating macrophages constitutively release basal levels of the pro-angiogenic cytokine VEGF. Our observation that in the absence of growth factors, cells of the MDA-MB-435s and MDA-MB-231 tumor lines release higher levels of VEGF than MCF-7 and MDA-MB-453 cells is consistent with the characterizations of these cell lines as models for late stage and early stage breast cancer respectively. MCF-7 is a poorly invasive cell line that is tumorigenic and expresses ER, which confers a requirement of estrogen for tumor formation [23], and e-cadherin,

the loss of which is correlated with aggressive breast cancers. MCF-7 cells lack vimentin [24] a mesenchymal marker and Her2/neu receptor which is correlated with poor prognosis in breast cancer [25]. On the other hand, the MDA-MB-435s cell line is a highly invasive cell line [23] that does not express either the ER or e-cadherin, but does express the mesenchymal marker vimentin [24]. Furthermore, higher levels of VEGF have been noted in the sera of patients with estrogen receptor-negative tumors that have similar surface marker profile to the MDA-MB-435s cell line than in patients with estrogen receptor-positive tumors that have surface marker profiles similar to MCF-7 cells [26]. These observations validate the use of the MCF-7 and the MDA-MB-435s cells lines as models for different stages of tumor progression, and we focused on these two cell lines to further elucidate the effects of CMTs on VEGF production.

In addition to basal levels of VEGF secretion, all the breast cancer cell lines examined responded to the angiogenic growth factor TGFβ by secreting increased levels of VEGF, whereas none of the cells responded to another pro-angiogenic factor IGF-I. Thus, TGFβ, which can induce angiogenesis directly and indirectly, specifically stimulated secretion of VEGF from all the breast tumor derived cells lines we investigated to various degrees, but not from the monocytic leukemia-derived cell line MonoMac 6. VEGF secretion from the weakly invasive breast cancer cell lines was stimulated to a lesser extent by TGFβ than from the more invasive cell lines. The increased responsiveness of the more aggressive cell lines may be due to a higher number of TGFβ receptors on these cells or the presence of multiple redundant pathways for VEGF secretion that TGFβ stimulates in these cells. This increased responsiveness of the more invasive cells to TGFβ probably gives them a higher angiogenic potential than the less invasive cells.

TGFβ did not increase secretion of another pro-angiogenic factor, bFGF, in any of these cells. bFGF lacks signal sequences for export through the classical secretory pathway, and appears to be released by either an ATP-driven peptide pump across the plasma membrane [27-29] by forming a complex with a carrier protein [30,31](184, 185) or alternatively through a calcium-dependent exocytic secretory pathway [32]. Whether TGFβ induces bFGF expression that leads to subsequent intracellular accumulation of this cytokine remains to be studied. Thus, TGFβ was identified as a selective dose-dependent inducer of VEGF secretion in the breast cancer cells.

As mentioned, neither TGFβ nor IGF-I caused an increase in VEGF secretion in MonoMac 6 cells. Vaday et al observed that TGFβ can suppress the effects of TNFα on MMP-9 expression in MonoMac 6 cells at a pre-translational level [33] indicating that MonoMac 6 cells possess receptors for TGFβ. Thus, the inability of TGFβ to induce VEGF secretion in MonoMac 6 cells is possibly due to the absence of specific pathways for VEGF induction by TGFβ rather than a lack of cellular responsiveness to TGFβ.

Precisely which pathway regulating VEGF production is responsive to TGFβ in the breast tumor cell lines is not clear. In mouse mammary epithelial cells, TGFβ cooperates with Ras to regulate VEGF synthesis [34,35]. In fibrosarcoma cells, TGFβ appears to act via an AP-1/HIF-1α-dependent pathway [36] and TGFβ may even synergize with the hypoxia pathway to stimulate VEGF production [37]. In aortic smooth muscle cells there is involvement of the p38 MAP Kinase pathway in TGFβ-induced VEGF production; [38] TGFβ also activates the p38 MAPK pathway and increases permeability in monolayers of endothelial cells [39]. The complexity of the actions of TGFβ is reflected in the growing consensus of the observations that either activation of the MAPK pathway or suppression of the SMAD pathway can increase the

pro-oncogenic effects of this growth factor which has previously been recognized primarily for its anti-oncogenic activity [40,41].

The inhibition of invasion and metastasis of tumor cells by CMTs was initially attributed predominantly to the anti-proteolytic properties of these tetracycline derivatives. We have identified a novel activity of CMTs that contributes to their antiangiogenic effectiveness. We have employed two CMTs – CMT 300 and CMT 308, both of which possess anti-proteolytic properties, to evaluate their capacity to inhibit angiogenic molecules.

Our results show that CMT 308 effectively reduced basal VEGF secretion in breast cancer cell lines as well as MonoMac 6 cells without accompanying cytotoxicity, reflecting an activity of this derivative on some step in a constitutive pathway for VEGF secretion in these cells. CMT 308 diminished basal VEGF release from MCF-7 and MDA-435s cells in a dose-dependent manner. Subcytotoxic doses of CMT 308 decreased basal VEGF secretion from the aggressively invasive MDA-MB-435s to a greater extent (80%) than from the less invasive MCF-7 cells (50%). CMT 308 was found to be an exceptionally potent inhibitor of constitutive VEGF release from MonoMac 6 cells. The virtually complete ablation of VEGF release into the medium of MonoMac 6 cells cultured in the presence of CMT 308 suggests that even if multiple pathways are involved in modulation of VEGF release by these monocytoid cells, these putative controls are sensitive to inhibition by CMT-308.

In addition to inhibiting basal VEGF secretion, CMT 308 also decreased TGFβ-induced VEGF secretion in the breast cancer cells. The average decrease in VEGF release from the poorly invasive cell lines MDA-MB-453 and MCF-7 (45%) in the simultaneous presence of TGFβ and CMT 308 was slightly lower than the average decrease in VEGF secretion from the aggressively invasive cell lines, MDA-MB-231 and MDA-MB-435s (60%), in the presence of the growth factor and the tetracycline derivative. Based on the results of a number of experimental tests, it appears that CMT 308 does not merely diminish the constitutive or basal levels of VEGF released by the tumor cell lines but also affects one or more of the pathways through which TGFβ can induce augmented VEGF secretion. However, because TGFβ-induced VEGF secretion is not completely suppressed by CMT 308 in breast cancer cells, the production of this growth factor may be controlled through CMT-sensitive as well as CMT-insensitive pathways. The pro-angiogenic activities of TGFβ are complex and occur through multiple, possible redundant pathways. At this stage we can only speculate which of these pathways for control of VEGF secretion are sensitive to modulation by CMT 308. Perhaps CMT 308 affects components of the p38 MAP Kinase pathway through which TGFβ induces VEGF. Alternatively, CMT 308 may affect the AP-1/HIF-1α pathway or components of the Ras oncogene pathway, which are all implicated in the activation of VEGF by TGFβ. Elucidation of the specific mechanism of action of CMT 308 and its targets will involve the dissection of these known pathways or identification of other, as yet unknown pathways.

Another pro-angiogenic cytokine, IGF-I, did not induce VEGF secretion in breast cancer cells, and CMT 308 reduced VEGF secretion from IGF-I treated cells to the same extent as from IGF-I untreated cells. Thus, in the breast cancer and monocytic cells that we examined, IGF-I does not induce VEGF and the presence of IGF-I does not affect basal VEGF inhibition by CMT 308. Whether other activities or pathways modulated by IGF-I are insensitive to CMT 308 will require further examination.

The inhibitory effects of CMT 308 in TGFβ or IGF-I treated MonoMac 6 cells remained the same in growth-factor treated and untreated cells. Since neither growth factor induced VEGF

secretion, CMT 308 appears to affect basal VEGF secretion in the MonoMac 6 cells. Thus, when cultured in serum-free medium, all pathways leading to VEGF secretion in MonoMac 6 cells appear to be sensitive to inhibition by CMT 308. In an interesting observation, VEGF secretion from MonoMac 6 cells treated with serum (FBS) was significantly higher than that from cells cultured in serum-free medium, and CMT 308 did not inhibit secretion of VEGF from MonoMac 6 cells grown in serum-containing medium. It is possible that components in serum such as growth factors induce augmented VEGF secretion through CMT-insensitive pathways. Indeed, VEGF released in the presence of serum may also be a result of synergistic activity of several growth factors and pathways, which subcytotoxic concentrations of CMT 308 are unable to inhibit. It is known that the CMT 300 binds to plasma proteins such as albumin [42]. Consequently, in the presence of serum-containing medium the tetracycline may not enter the cells. It is recognized that CMTs enter the target cell (S. Kocer, S R. Simon, unpublished data). Thus, the loss of effectiveness of the CMT in the presence of serum, and its subsequent inability to enter the cell indicates that the effects of CMT 308 on the tumor cells occur intracellularly rather than extracellularly.

The time course of the effects of CMT 308 on VEGF secretion by MCF-7 cells suggests that the apparent potency of CMT 308 in this breast tumor cell line, is a function of inhibitor dose as well as time in the presence of the inhibitory agent. MCF-7 cells had an apparent capacity to escape from the suppressive effects of CMT 308 after 8 hours of exposure to CMT 308. The resumption of VEGF secretion seen after 8 hours may be due to progressive loss of sensitivity of one or more of the pathways regulating VEGF secretion to inhibition by CMT 308, or alternatively to the emergence of a new, CMT-resistant pathway for modulating VEGF secretion. Neither of these potential mechanisms can be attributed simply to the total length of time that the tumor cells are maintained in culture, as evidenced by the observation that secretion of new VEGF is still suppressed for approximately eight hours even if the CMT is not added to freshly established cultures for several hours. Significantly, the levels of pre-existing VEGF are not diminished by addition of CMT 308, suggesting that the tetracycline derivative does not trigger a pathway for destruction of the growth factor. We conclude that only synthesis of VEGF is affected, but the mechanism for temporal control of inhibition is still elusive. Because we suspect that the CMTs have an intracellular site of action, the temporally limited inhibition by CMT 308 may reflect a time-dependent capacity of the cells to clear the agent from the cytosol. Possible clearance mechanisms might include catabolism of the CMT by the cells, or, alternatively, enhanced efflux of the CMT from the cell. It is unlikely, however that the drug itself spontaneously loses potency over time in serum-free medium, since in MonoMac 6 cells CMT 308 remains a potent inhibitor of VEGF secretion up to 48 hours.

We observed that as the potency of CMT 308 in the medium decreased over time, the ability of TGFβ to enhance VEGF secretion increased. The apparently compromised ability of cells to respond to TGFβ in the presence of CMT 308 suggests that the tetracycline inhibits not only constitutive secretion of VEGF but also at least part of the TGFβ-regulated pathway which results in augmented VEGF secretion. If TGFβ were able to induce increased VEGF secretion via a mechanism which was completely insensitive to the presence of CMT 308, the increment in the levels of VEGF which were secreted after the addition of a fixed amount of TGFβ for a fixed amount of time would be constant, regardless of how much inhibition of basal VEGF secretion by CMT 308 could be achieved. In fact, in our studies of temporal dependence of TGFβ-induced VEGF augmentation in cultures in which the inhibitory effects of CMT 308 were decaying, we

observed that the increment in the levels of VEGF secreted after the addition of a fixed amount of TGFβ was smaller when the residual potency of CMT 308 as an inhibitor was greater. Thus, cells stimulated with TGFβ in the presence of CMT 308 could only increase VEGF levels to the extent that the declining activity of the tetracycline would permit. It is premature from this data to argue that there is no portion of the control mechanism for VEGF secretion which is subject to TGFβ-mediated augmentation but is insensitive to CMT 308; however, we feel confident that CMT 308 blocks at least some of the TGFβ-mediated augmentation.

In contrast to the effects of CMT-308 on breast tumor cell lines, the kinetics of inhibition of VEGF release from MonoMac 6 cells indicate that the activity of CMT 308 is more prolonged in this cell line: in the presence of the tetracycline derivative there is continuous inhibition of VEGF secretion for at least 24 hours. VEGF secretion by MonoMac 6 cells is insensitive to growth factors (TGF or IGF-1), suggesting that VEGF release in these cells is controlled by a single pathway which is targeted by CMT 308. If there is any modulation of VEGF secretion beyond the constitutive activity seen in MonoMac 6 cells, we have not uncovered any natural mediator that can increase or diminish production of the angiogenic growth factor. It can only be speculated whether the sustained inhibition of VEGF release by MonoMac 6 cells in the presence of CMT 308 is mechanistically related to the insensitivity of the cell line to modulation of VEGF release by TGFβ or IGF-I.

While Seftor et al have observed that CMT 300 decreased mRNA levels for VEGF-C in human cutaneous and uveal melanoma cell lines [43], in the breast cancer cell lines that we used, the reduction in secreted VEGF produced by CMT 308 was not a result of inhibition of transcription of the mRNA species for the $VEGF_{121}$ and $VEGF_{165}$ mRNA isoforms. The variations in the VEGF mRNA species isolated from the different cell lines used may account for the apparent differences in the mechanism of modulation of VEGF production. We also did not observe changes in the concentrations of the different VEGF mRNA species upon treatment of cells with TGFβ, although TGFβ has been shown to increase VEGF mRNA levels in several other cell types such as osteoblasts [44], fibroblasts [45], keratinocytes [46] and breast tumor cells [12] by inducing transcription of the VEGF gene. Although the levels of the multiple VEGF mRNAs we visualized were unaltered in MCF-7 and MDA-MB-435s cells upon addition of TGFβ, we did observe enhanced secretion of VEGF by both cell lines in the presence of the growth factor. The apparent absence of modulation of VEGF mRNAs at the transcriptional level by addition of TGFβ to the breast tumor cell lines we have studied is paralleled by a similar failure of CMT 300 or CMT 308 to alter levels of VEGF mRNAs in these tumor cell lines, either in the presence or absence of TGFβ. Our results are consistent with the report that CMT 300 does not inhibit transcription of the inducible Nitric Oxide Synthase (iNOS) gene in murine macrophages [48,49] nor does it affect COX-2 mRNA in murine macrophages [50]. nor does it affect COX-2 mRNA in murine macrophages through an as yet uncharacterized post-transcriptional regulatory mechanism [49]. We observed that neither CMT 300 nor CMT 308 affected alternative splicing of VEGF mRNA in the breast cancer cell lines we examined, an event that occurs post-transcriptionally.

In MonoMac 6 cells, levels of two mRNA species which presumably code for the two isoforms of VEGF protein were diminished slightly in the presence of 5 μM CMT 300. This may be indicative of some inhibition of VEGF transcription by CMT 300. However, this does not correlate with the failure of CMT-300 to diminish either VEGF release or intracellular levels of VEGF. In contrast to CMT 300, CMT 308 does not significantly decrease VEGF mRNA

levels in Mono Mac 6 cells. This indicates that the most likely effects of CMT 308 on VEGF production by MonoMac 6 cells as well as the breast tumor cell lines we have studied target some post-transcriptional events, perhaps at the level of secretion.

The reduction in levels of intracellular pools of VEGF, but not of VEGF mRNA levels in breast cancer cells treated with CMT 308 indicates that CMT 308 may affect post-transcriptional events in these cells. CMTs may affect cytoplasmic accumulation of VEGF by inhibiting translation of VEGF protein without affecting transcription. Alternatively, CMTs may degrade intracellular VEGF protein upon translation. Although CMT 308 does not cause a generalized reduction in total protein levels in these cells, specific degradation of cytoplasmic VEGF may be possible, perhaps by targeted degradation through the ubiquitin-proteasome pathway. Additionally, it is possible that CMT 308 induces a redistribution of intracellular VEGF into different compartments such as the ER where glycosylation of VEGF and folding normally take place [51]. Since proteins that are misfolded are often retained in the ER and then delivered to the proteasome to be degraded, it may be possible that CMT 308 causes a post-translational misfolding of a selected subset of proteins including VEGF, as suggested by Patel et al [50]. Misfolded proteins are generally targeted for degradation via the ubiquitin pathway, leading to selective reductions in steady-state levels of the specific proteins. The relatively low cytoplasmic levels of VEGF compared to the extracellular levels of the growth factor in conditioned media from tumor cell lines indicate that the VEGF protein is efficiently secreted rather than stored intracellularly. Treatment with TGFβ increases cytoplasmic VEGF as well as secretion of VEGF, consistent with a model in which TGFβ increases translation of VEGF protein without increasing levels of VEGF mRNA. This may be achieved by increasing the availability or efficiency of translational machinery. Other examples of control of protein expression by modulating levels of factors which control protein synthesis have been described previously. It has been observed that long-term potentiation in dendrites of neuronal cells in the hippocampus may reflect augmented translation of existing mRNA pools in dendrites which is ascribed to increased functional activity of a critical translation factor eIF-4E (eukaryotic translation initiation factor-4E) [52]. Another group observed that calreticulin (CRT) can interact with a CUG triplet repeat binding protein (CUGBP1) and thereby induce increased levels of transcription factors by augmenting the rates at which the proteins are synthesized [53]. In another example of post-transcriptional control of regulatory molecule synthesis, it was observed that expression of C/EBPα, a transcriptional regulator is suppressed at the translational level by interaction of the poly(rC)-binding protein hnRNP E2 with *CEBPA* mRNA [55]. We may hypothesize that, in a fashion analogous to these examples, TGFβ could induce the expression or availability of translational regulatory factors that could in turn contribute to the augmentation of intracellular levels of VEGF protein in MCF-7 cells.

It has been reported that different cell types may express different molecular weight isoforms of VEGF, some of which appear to be novel species associated with specific cell types. In our studies, we reported that MonoMac 6 cells appear to secrete several isoforms of VEGF, some of which are distinct from those observed by others [56-58]. This unique isoform appears to be the species whose secretion is sensitive to inhibition by CMT 308. Further elucidation of the characteristics of this isoform and its secretory pathway may help us understand better the specific targets for CMT 308.

We have considered whether the capacity of CMT 308 to lower levels of VEGF in the conditioned medium of the cell lines we investigated may be linked mechanistically to the

metalloprotease inhibitory activity of the tetracycline derivative. It has been observed that matrix metalloprotease-9 (MMP-9) can trigger the angiogenic switch *in vivo* by increasing the availability of VEGF in very early stages of pancreatic tumors [59]. If inhibition of MMP-9 were the main cause for the reduction in VEGF secretion, however, we would have expected to observe comparable diminutions in VEGF after treatment with CMT 300 and CMT 308, which are both potent inhibitors of MMP-9 [54,60]. Also, human glioma cells that are transfected with membrane-type I MMP (MT-I MMP) secrete greater levels of VEGF in the conditioned media than control cells. This increase in secreted VEGF could be downregulated by addition of GM6001, a general MMP inhibitor [61]. This data indicates that inhibitors of MT-I MMP may down-regulate secretion of VEGF by tumor cells. CMT 300 has been shown to inhibit MT-I MMP activation of pro-MMP-2 in COS-1 cells and to decrease invasiveness in HT1080 fibrosarcoma cells [62]. However, our results with MCF-7 and MDA-MB-435s cells show that CMT 300 is not an effective inhibitor of VEGF release. This makes it unlikely that the diminished levels of secreted VEGF in cells treated with CMT 308 can be attributed to the established capacity of tetracyclines to inhibit metalloprotease activities. More specifically, the inhibition of VEGF release mediated by CMT-308 does not appear to be a result of inhibition of soluble MMPs or MT-I MMP.

CMT 300, which lacks an amino group on the 9-position of the tetracycline ring system but is otherwise identical in chemical structure to CMT-308, has a modest stimulatory effect on basal levels of VEGF released by MCF-7 cells. CMT 300 has an even more marked dose-dependent stimulatory effect on the sizes of both, he cytosolic and extracellular pools of VEGF produced in MDA-MB-435s cells, in the absence as well as the presence of TGFβ. CMT 300 at concentrations higher than 5 μM stimulated mitochondrial dehydrogenase activity in these cells, as detected by the MTS assay, suggesting non-specific activation of the MDA-MB-435s cells. This apparent activation by CMT 300 was also observed in MCF-7 cells, as well as MonoMac 6 cells. Based on the results of our cytotoxicity studies on the breast cancer cell lines and MonoMac 6 cells, we speculate that the stimulation of VEGF release from cells cultured in the presence of low doses of CMT 300 may be a result of a non-specific activation response. Tetracyclines are known to affect mitochondrial protein synthesis, and effects of CMT 300, but not its derivatives, on mitochondrial membrane potential in prostate tumor cell lines have been described previously [63]. CMT 300 has a modest stimulatory effect on basal levels of VEGF released by MCF-7 cells, while in MDA-MB-435s cells the tetracycline derivative has a more marked dose-dependent stimulatory effect on basal VEGF release. In fact, CMT 300 consistently stimulates VEGF release from breast cancer cell lines up to 20 μM. We have found no conditions under which CMT-300 has an inhibitory effect on basal VEGF levels in these cells, given their relative resistance to any cytotoxic effects of this agent. In addition, culture of cells in the presence of CMT 300 does not result in diminution in the levels of intracellular VEGF protein isoforms or VEGF mRNA species in the absence of significant cytotoxicity in the breast cancer cells. We conclude that CMT 300 is not an inhibitor of VEGF release in these cells. The differences between the effects of CMT 300 and 308 on VEGF secretion in breast tumor cell lines may be attributed to the presence of the amino group on the $9^{th}$ carbon atom in CMT 308, but the detailed mechanisms by which this tetracycline derivative causes diminished secretion of VEGF remain to be elucidated. The apparent reduction of VEGF release in Mono Mac 6 cells when treated with doses of CMT 300 higher than 5 μM may reflect cytotoxicity, but

like the dramatic inhibition of VEGF release in these cells caused by CMT 308, may also reflect additional cell-specific effects of the CMT on secretion of VEGF.

During the angiogenesis process, endothelial cells undergo morphological changes, and form tube-like structures that are ultimately strengthened and stabilized by smooth muscle cells (pericytes). Components of the normal signaling mechanisms that result in the morphogenic transition of endothelial cells to form neovessels appear to arise from interactions of the cells with the surrounding extracellular matrix. Matrigel is a basement membrane matrix comprising mainly of laminin, collagen IV, and heparan sulfate proteoglycans along with several growth factors. Unlike thinner layers of Matrigel on which endothelial cells grow to confluence, Matrigel in a thick layer promotes spontaneous tube formation by endothelial cells, presumably induced in part by the endogenous growth factors [8]. Depletion of the growth factors is reported by the manufacturer to result in less tube formation, while replacement with putative pro-angiogenic agents restores robust tube formation. There is currently no evidence that the tubular networks formed by endothelial cells in Matrigel have lumens or progress to functional microvessels. Nevertheless, the capacity to undergo analogous morphological changes favoring vessel formation is an integral part of early angiogenesis. It should be noted that virtually all known anti-angiogenic drugs tested by B.D Biosciences, the manufacturer of Matrigel, inhibit endothelial tube formation in this basement membrane-like matrix to variable extents, although some agents appear to achieve only partial inhibition even at the highest doses tested.

Doses of CMT 300 and CMT 308 in the low micromolar range both inhibited the morphological changes leading to tube formation by HMVEC grown on Matrigel. The tubes formed by HMVEC appeared to be multicellular structures bridging separated islands or clumps of cells. This inhibition was not associated with cytotoxicity and cells remained viable when treated with the CMTs. If cells were treated with the CMTs, their capacity to form tubes on thick layers of Matrigel was diminished: the tubes were significantly reduced in thickness, and fewer bridges were formed. These reductions in the features of the morphogenic transition induced by Matrigel were more pronounced when cells had been exposed to higher concentrations of the CMTs. We then examined the ability of endothelial cells to form similar tubes when grown only on Collagen I. As has been shown by Whelan and Senger [9], endothelial cells spontaneously undergo a transition to a reticular network-like morphology when grown with collagen I. When the cells were exposed to CMT 300 and CMT 308 their capacity to undergo this type I collagen-induced morphogenic transition was also compromised in a dose-dependent fashion. The branches between islands of cells that were multicellular and thick when cells were exposed to collagen in the absence of the CMTs were thinner unicellular and were diminished in number when CMT 300- or CMT 308-treated cells were exposed to type I collagen. CMT 308 appeared to be slightly more effective than CMT 300 at the same concentrations in downregulating the collagen-induced morphogenic transition. The inhibition of the collagen-induced morphogenic transition by CMTs while clearly significant appeared to be less dramatic than the inhibition of tube formation by cells grown on Matrigel, indicating that perhaps in the presence of a full complement of basement membrane extracellular matrix proteins endothelial cells may be more sensitive to inhibition of tube formation than in the presence of a single stromal matrix protein. Whelan et al had shown that the formation of the network of cord-like structures formed by HMVEC on type I collagen involved the ligation of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins [9]. This was followed by suppression of cAMP and cAMP-dependent protein kinase, leading to changes in actin polymerization. It is possible that CMT 300 and CMT 308 either affect the engaging of

integrins, or inhibit the suppression of cAMP or the protein kinase that are involved in the morphogenic transition in HMVEC [9]. In order to determine whether the morphological changes could be reversed after formation of the tubes, CMTs were added after tubes were formed. No change was observed in existing tubes formed either on Matrigel or on collagen I upon treatment with CMTs. There was neither any obvious diminution in the numbers of branches between islands nor was there any apparent reduction in their thickness. It appears that once the morphological changes occur, CMTs are unable to reverse these changes. Thus, the data indicate that the targets for inhibition of morphological change by CMTs may be pathways involved in the initiation of shape change. At none of the doses of CMT 300 and CMT 308 used, did calcein fluorescence of viable endothelial cells disappear, indicating that loss of tube formation was not associated with cytotoxicity. In addition, an MTS assay performed on similarly treated HMVEC also indicated that inhibition of tube formation was not associated with cytotoxicity. The observation that CMTs are not cytotoxic to normal primary endothelial cells *in vitro* may be important in the development of these agents as inhibitors of angiogenesis *in vivo*, because their use may be associated with fewer side-effects in patients undergoing therapy.

We can only speculate at this time whether the previously documented inhibition of matrix metalloproteases by CMTs has any role in the inhibition of morphogenic transitions in endothelial cells. HMVEC treated with CMT 5 were prevented from undergoing this morphogenic change just as they were with CMT 300 and CMT 308. CMT 5 (tetracycline pyrazole) is a modified tetracycline, whose $Ca^{2+}$ and $Zn^{2+}$ binding sites are blocked by the pyrazole ring [64]. The metalloprotease inhibitory activity of CMTs has been attributed predominantly to their $Ca^{2+}$ and $Zn^{2+}$ binding capacity, and indeed in assays of MMP inhibitory activity *in vitro*, CMT 5 appears to be inactive [65]. The capacity of CMT 5 to inhibit tube formation in endothelial cells presents the possibility that the mechanism of action of the CMTs in diminishing the morphogenic transition involves a cellular events other than proteolysis of the surrounding extracellular matrix. It is possible that the portion of the modified tetracycline molecule that is responsible for inhibiting the morphological changes in HMVEC lies in a region that is common to CMT 300, CMT 308 and CMT 5. This would represent a unique activity of CMTs independent of their capacity to inhibit MMPs.

Since the CMTs could affect morphologic changes in endothelial cells, which lead to formation of networks or tubes, we evaluated their capacity to inhibit migration of endothelial cells towards a chemoattractant. The process of migration is generally considered to require morphologic changes as a component of cellular movement. Our assay for HUVEC migration employs fibronectin-coated porous membrane bottomed cell culture inserts as barriers. Because the fibronectin coating ensures that the endothelial cells remain adherent but does not occlude the pores on the membrane bottoms of the inserts, migration through the pores can proceed without the involvement of proteases such as matrix metalloproteases, which are known to be secreted by endothelial cells when invading through a basement membrane. CMT 308 inhibited migration of HUVEC through these porous inserts, presumably reflecting a mechanism of inhibition that is independent of protease activity. This is consistent with the effects of CMTs on tube-formation of HMVEC lending further support to the hypothesis that the CMTs may affect the capacity of endothelial cells to undergo changes in shape that are essential for neovessel formation and cellular migration. The underlying mechanisms for such shape changes may be independent of processes that involve matrix metalloproteases.

CMT 300 as well as CMT 308 inhibited invasion of HUVEC through Matrigel towards VEGF as a chemoattractant. The CMTs did not inhibit invasion in the absence of a chemoattractant indicating that the CMTs may simply inhibit the movement of HUVEC directly or may inhibit the response of the cells to VEGF. Yao et al observed that minocycline, a synthetic tetracycline inhibited VEGF-induced migration of smooth muscle cells possibly via the PI3 Kinase/AKt pathway. They also observed that minocycline inhibited MMP-9 through down-regulation of ERK1/2 activation [47]. In our experiments with endothelial cells, inhibition of migration and tube formation by CMTs may also be attributed the down-regulation of the ERK1/2 –mediated MMP-9 activation or PI3K-mediated migration. Whether these pathways are involved in the migratory processes and tube formation in endothelial cells and whether these are inhibited by CMTs need further examination.

The conditioned medium of MonoMac 6 cells cultured in serum-free medium appears to contain chemoattractants released by the cells which induce invasion of HMVEC through Matrigel-coated membranes. In a somewhat unexpected observation, conditioned medium from MonoMac 6 cells treated with CMT 308 displayed undiminished chemoattractant activity towards HMVEC invasion, whereas medium from CMT 300-treated cells showed statistically significant diminished chemoattractant activity. MonoMac 6 cells treated with lower doses of CMT 300 were observed to release augmented levels of VEGF, whereas treatment of the cells with CMT 308 resulted in marked diminution of VEGF release. The combination of residual CMT 308 and the extremely low levels of VEGF in the conditioned medium of CMT 308-treated MonoMac 6 cells would be expected to induce relatively little HMVEC invasion relative to the chemoattractant activity in the conditioned medium of mock-treated cells if in fact VEGF were the only chemoattractant released by the cultured cells. The apparently undiminished chemoattractant activity in the conditioned medium of CMT 308-treated MonoMac 6 cells may reside in other chemokines released by the cells which are not subject to inhibition by CMTs. It will be recalled that release of the neutrophil chemokine, IL-8, by these cells is not affected by CMTs. It is possible that the diminished invasion of HMVEC towards conditioned medium of CMT 300-treated cells is a direct result of diffusion of the CMT to the upper chamber and inhibition of changes in endothelial cell morphology independent of the levels of chemoattractant activity in the conditioned medium. A more thorough examination of the effects of dilution of the MonoMac 6 conditioned medium so that the concentration of CMTs in the diluted medium would fall below the levels which induce inhibition of endothelial cell shape changes would clarify the origins of augmented or diminished chemoattractant activity in the supernatant medium of MonoMac 6 cells cultured under different conditions.

In conclusion, CMT 300 and CMT 308 inhibit the morphogenic transitions in endothelial cells which normally result in tube-like structures in Matrigel and reticular networks in type I collagen in a dose-dependent manner, and decrease migration and invasion of HMVEC towards VEGF to variable degrees. These results offer evidence that the CMTs can directly inhibit endothelial cell changes that are required for new microvessel formation or angiogenesis.

The work summarized in this report has demonstrated a novel anti-angiogenic capacity for CMT 300 and CMT 308. Future studies focusing on the molecular mechanisms of inhibition of VEGF production by CMT 308 and the mechanisms of morphological changes in endothelial cells which are sensitive to inhibition by CMTs will further our understanding of the functioning of this group of modified tetracyclines. In addition, this work provides preclinical data which

can serve as a basis for *in vivo* and clinical studies with CMT 308 as a non-phototoxic alternative to CMT 300 in the management of angio-proliferative cancers.

**F. References**

1. Cianfrocca, M, Cooley, TP, Lee, JY, Rudek, MA, Scadden, DT, Ratner, L, Pluda, JM, Figg, WD, Krown, SE, and Dezube, BJ 2002 Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS melignancy consortium study. *J. Clin. Oncol. 20:153.*
2. Seftor, RE, Seftor, EA, Kirschmann, DA, and Hendrix, MJ 2002 Targeting the tumor microenvironment with chemically modified tetracyclines: inhibition of laminin 5 gamma2 chain promigratory fragments and vasculogenic mimicry. *Mol. Cancer Therapeut. 1:1173.*
3. Fife, RS, Sledge, GW Jr, Sissons, S, and Zerler, B 2000 Effects of tetracyclines on angiogenesis *in vitro*. *Cancer Lett. 153:75*.
4. Rudek, MA, Figg, WD, Dyer, WV, Dahut, W, Turner, ML, Steinberg, SM, Liewehr, DJ, Kohler, DR, Pluda, JM, and Reed, E 2001 Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. *J. Clin. Oncol. 19:584*.
5. Zajchowski, DA, Bartholdi, MF, Gong, Y, Webster, L, Liu, H-L, Munishkin, A, Beauheim, C, Harvey, S, Ethier, SP, and Johnson, PH 2001 Identificaiton of gene expression profiles that predict the aggressive behavior of breast cancer cells. *Cancer Res. 61:5168.*
6. Price, JE, and Zhang, RD 1990 Studies of human breast cancer metastasis using nude mice. *Cancer Metastasis Rev. 8:285*.
7. Munaut, C, Noel, A, Hougrand, O, Foidart, J-M, Boniver, J, and Deprez, M 2003 Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2, and MMP-9 in human glioblastomas. *Int. J. Cancer 106:848*.
8. Kubota, Y, Kleinman, HK, Martin, GR, and Lawley, TJ 1988 Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol. 107:1589.*
9. Whelan, MC, and Senger, DR 2003 Collagen I initiates endothelial cell morphogenesis by inducing actin polymerization through suppression of cyclic AMP and protein kinase A. *J. Biol. Chem. 278:327.*
10. Simon, SR, and Kothari, M 2002 Antiangiogenic action of chemically modified tetracyclines in breast cancer. *Department of Defense Breast Cancer Research Program Meeting: Era of Hope. Proceedings, Vol III, P46-19.*
11. Kong, F. M., M. S. Anscher, T. Murase, B. D. Abbott, J. D. Iglehart, and R. L. Jirtle. 1995. Elevated plasma transforming growth factor-beta 1 levels in breast cancer patients decrease after surgical removal of the tumor. *Ann Surg 222:155*.
12. Donovan, D., J. H. Harmey, D. Toomey, D. H. Osborne, H. P. Redmond, and D. J. Bouchier-Hayes. 1997. TGF beta-1 regulation of VEGF production by breast cancer cells. *Ann Surg Oncol 4:621*.
13. Saadeh, P. B., B. J. Mehrara, D. S. Steinbrech, M. E. Dudziak, J. A. Greenwald, J. S. Luchs, J. A. Spector, H. Ueno, G. K. Gittes, and M. T. Longaker. 1999. Transforming growth factor-beta1 modulates the expression of vascular endothelial growth factor by osteoblasts. *Am J Physiol 277:C628.*
14. Pepper, M. S. 1997. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev 8:21*.

15. Pertovaara, L., A. Kaipainen, T. Mustonen, A. Orpana, N. Ferrara, O. Saksela, and K. Alitalo. 1994. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem 269:6271*.
16. Gerwins, P., E. Skoldenberg, and L. Claesson-Welsh. 2000. Function of fibroblast growth factors and vascular endothelial growth factors and their receptors in angiogenesis. *Crit Rev Oncol Hematol 34:185*.
17. Akagi, Y., W. Liu, B. Zebrowski, K. Xie, and L. M. Ellis. 1998. Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I. *Cancer Res 58:4008*.
18. Bermont, L., F. Lamielle, S. Fauconnet, H. Esumi, A. Weisz, and G. L. Adessi. 2000. Regulation of vascular endothelial growth factor expression by insulin-like growth factor-I in endometrial adenocarcinoma cells. *Int J Cancer 85:117*.
19. Grimberg, A., and P. Cohen. 2000. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol 183:1*.
20. Klein, S., M. Roghani, and D. B. Rifkin. 1997. Fibroblast growth factors as angiogenesis factors: new insights into their mechanism of action. *Exs 79:159*.
21. Kroon, A. M., B. H. Dontje, M. Holtrop, and C. Van den Bogert. 1984. The mitochondrial genetic system as a target for chemotherapy: tetracyclines as cytostatics. *Cancer Lett 25:33*.
22. van den Bogert, C., B. H. Dontje, and A. M. Kroon. 1985. The antitumour effect of doxycycline on a T-cell leukaemia in the rat. *Leuk Res 9:617*.
23. Rochefort, H., N. Platet, Y. Hayashido, D. Derocq, A. Lucas, S. Cunat, and M. Garcia. 1998. Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview. *J Steroid Biochem Mol Biol 65:163*.
24. Sommers, C. L., S. W. Byers, E. W. Thompson, J. A. Torri, and E. P. Gelmann. 1994. Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res Treat 31:325*.
25. Tari, A. M., S. J. Lim, M. C. Hung, F. J. Esteva, and G. Lopez-Berestein. 2002. Her2/neu induces all-trans retinoic acid (ATRA) resistance in breast cancer cells. *Oncogene 21:5224*.
26. Heer, K., H. Kumar, J. R. Read, J. N. Fox, J. R. Monson, and M. J. Kerin. 2001. Serum vascular endothelial growth factor in breast cancer: its relation with cancer type and estrogen receptor status. *Clin Cancer Res 7:3491*.
27. Klionsky, D. J., R. Cueva, and D. S. Yaver. 1992. Aminopeptidase I of Saccharomyces cerevisiae is localized to the vacuole independent of the secretory pathway. *J Cell Biol 119:287*.
28. Kurokawa, T., R. Sasada, M. Iwane, and K. Igarashi. 1987. Cloning and expression of cDNA encoding human basic fibroblast growth factor. *FEBS Lett 213:189*.
29. von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem 133:17*.
30. Florkiewicz, R. Z., R. A. Majack, R. D. Buechler, and E. Florkiewicz. 1995. Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. *J Cell Physiol 162:388*.
31. Piotrowicz, R. S., J. L. Martin, W. H. Dillman, and E. G. Levin. 1997. The 27-kDa heat shock protein facilitates basic fibroblast growth factor release from endothelial cells. *J Biol Chem 272:7042*.

32. Mignatti, P., T. Morimoto, and D. B. Rifkin. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J Cell Physiol 151:81*.
33. Vaday, G. G., H. Schor, M. A. Rahat, N. Lahat, and O. Lider. 2001. Transforming growth factor-beta suppresses tumor necrosis factor alpha- induced matrix metalloproteinase-9 expression in monocytes. *J Leukoc Biol 69:613*.
34. Breier, G., S. Blum, J. Peli, M. Groot, C. Wild, W. Risau, and E. Reichmann. 2002. Transforming growth factor-beta and Ras regulate the VEGF/VEGF-receptor system during tumor angiogenesis. *Int J Cancer 97:142*.
35. Janda, E., K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, and S. Grunert. 2002. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol 156:299*.
36. Shih, S. C., and K. P. Claffey. 2001. Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. *Growth Factors 19:19*.
37. Sanchez-Elsner, T., L. M. Botella, B. Velasco, A. Corbi, L. Attisano, and C. Bernabeu. 2001. Synergistic cooperation between hypoxia and transforming growth factor- beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem 276:38527*.
38. Yamamoto, T., O. Kozawa, K. Tanabe, S. Akamatsu, H. Matsuno, S. Dohi, and T. Uematsu. 2001. Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. *J Cell Biochem 82:591*.
39. Goldberg, P. L., D. E. MacNaughton, R. T. Clements, F. L. Minnear, and P. A. Vincent. 2002. p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. *Am J Physiol Lung Cell Mol Physiol 282:L146*.
40. Saha, D., P. K. Datta, and R. D. Beauchamp. 2001. Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. *J Biol Chem 276:29531*.
41. Wakefield, L. M., and A. B. Roberts. 2002. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev 12:22*.
42. Rudek, M. A., J. Venitz, Y. Ando, E. Reed, J. M. Pluda, and W. D. Figg. 2003. Factors involved in the pharmacokinetics of COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer: clinical and experimental studies. *J Clin Pharmacol 43:1124*.
43. Seftor, R. E., E. A. Seftor, J. E. De Larco, D. E. Kleiner, J. Leferson, W. G. Stetler-Stevenson, T. F. McNamara, L. M. Golub, and M. J. Hendrix. 1998. Chemically modified tetracyclines inhibit human melanoma cell invasion and metastasis. *Clin Exp Metastasis 16:217*.
44. Saadeh, P. B., B. J. Mehrara, D. S. Steinbrech, M. E. Dudziak, J. A. Greenwald, J. S. Luchs, J. A. Spector, H. Ueno, G. K. Gittes, and M. T. Longaker. 1999. Transforming growth factor-beta1 modulates the expression of vascular endothelial growth factor by osteoblasts. *Am J Physiol 277:C628*.
45. Pertovaara, L., A. Kaipainen, T. Mustonen, A. Orpana, N. Ferrara, O. Saksela, and K. Alitalo. 1994. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem 269:6271*.
46. Frank, S., G. Hubner, G. Breier, M. T. Longaker, D. G. Greenhalgh, and S. Werner. 1995. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem 270:12607*.

47. Yao, J. S., Y. Chen, W. Zhai, K. Xu, W. L. Young, and G. Y. Yang. 2004. Minocycline exerts multiple inhibitory effects on vascular endothelial growth factor-induced smooth muscle cell migration: the role of ERK1/2, PI3K, and matrix metalloproteinases. *Circ Res 95:364*.
48. Trachtman, H., S. Futterweit, R. Greenwald, S. Moak, P. Singhal, N. Franki, and A. R. Amin. 1996. Chemically modified tetracyclines inhibit inducible nitric oxide synthase expression and nitric oxide production in cultured rat mesangial cells. *Biochem Biophys Res Commun 229:243*.
49. Amin, A. R., M. Attur, and S. B. Abramson. 1999. Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. *Curr Opin Rheumatol 11:202*.
50. Patel, R. N., M. G. Attur, M. N. Dave, I. V. Patel, S. A. Stuchin, S. B. Abramson, and A. R. Amin. 1999. A novel mechanism of action of chemically modified tetracyclines: inhibition of COX-2-mediated prostaglandin E2 production. *J Immunol 163:3459*.
51. Vega, V. L., and A. De Maio. 2003. Geldanamycin treatment ameliorates the response to LPS in murine macrophages by decreasing CD14 surface expression. *Mol Biol Cell 14:764*.
52. Cracco, J. B., P. Serrano, S. I. Moskowitz, P. J. Bergold, and T. C. Sacktor. 2005. Protein synthesis-dependent LTP in isolated dendrites of CA1 pyramidal cells. *Hippocampus*.
53. Timchenko, N. A., G. L. Wang, and L. T. Timchenko. 2005. CUG triplet repeat binding protein, CUGBP1, increases translation of C/EBP beta isoform, LIP, by interacting with the alpha and beta subunits of eIF2. *J Biol Chem*.
54. Ramamurthy, N. S., B. R. Rifkin, R. A. Greenwald, J. W. Xu, Y. Liu, G. Turner, L. M. Golub, and A. T. Vernillo. 2002. Inhibition of matrix metalloproteinase-mediated periodontal bone loss in rats: a comparison of 6 chemically modified tetracyclines. *J Periodontol 73:726*.
55. Perrotti, D., V. Cesi, R. Trotta, C. Guerzoni, G. Santilli, K. Campbell, A. Iervolino, F. Condorelli, C. Gambacorti-Passerini, M. A. Caligiuri, and B. Calabretta. 2002. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. *Nat Genet 30:48*.
56. Munaut, C., A. Noel, O. Hougrand, J. M. Foidart, J. Boniver, and M. Deprez. 2003. Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. *Int J Cancer 106:848*.
57. Connolly, J. M., and D. P. Rose. 1998. Angiogenesis in two human prostate cancer cell lines with differing metastatic potential when growing as solid tumors in nude mice. *J Urol 160:932*.
58. Eckhart, L., J. Ban, C. Ballaun, W. Weninger, and E. Tschachler. 1999. Reverse transcription-polymerase chain reaction products of alternatively spliced mRNAs form DNA heteroduplexes and heteroduplex complexes. *J Biol Chem 274:2613*.
59. Bergers, G., R. Brekken, G. McMahon, T. H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, and D. Hanahan. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol 2:737*.
60. Greenwald, R. A., L. M. Golub, N. S. Ramamurthy, M. Chowdhury, S. A. Moak, and T. Sorsa. 1998. In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. *Bone 22:33*.
61. Deryugina, E. I., L. Soroceanu, and A. Y. Strongin. 2002. Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis. *Cancer Res 62:580*.
62. Lee, H. M., L. M. Golub, J. Cao, O. Teronen, M. Laitinen, T. Salo, S. Zucker, and T. Sorsa. 2001. CMT-3, a non-antimicrobial tetracycline (TC), inhibits MT1-MMP activity: relevance to cancer. *Curr Med Chem 8:257*.

63. Kroon, A. M., B. H. Dontje, M. Holtrop, and C. Van den Bogert. 1984. The mitochondrial genetic system as a target for chemotherapy: tetracyclines as cytostatics. *Cancer Lett 25:33*.
64. Liu, Y., N. Ramamurthy, J. Marecek, H. M. Lee, J. L. Chen, M. E. Ryan, B. R. Rifkin, and L. M. Golub. 2001. The lipophilicity, pharmacokinetics, and cellular uptake of different chemically-modified tetracyclines (CMTs). *Curr Med Chem 8:243*.
65. Sorsa, T., N. S. Ramamurthy, A. T. Vernillo, X. Zhang, Y. T. Konttinen, B. R. Rifkin, and L. M. Golub. 1998. Functional sites of chemically modified tetracyclines: inhibition of the oxidative activation of human neutrophil and chicken osteoclast pro- matrix metalloproteinases. *J Rheumatol 25:975*.
66. Houck, K. A., D. W. Leung, A. M. Rowland, J. Winer, and N. Ferrara. 1992. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem 267:26031*.
67. Park, J. E., G. A. Keller, and N. Ferrara. 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell 4:1317*.

# Table 1

| Cell Line | Properties | Basal VEGF Secretion (pg/ml) |
|---|---|---|
| MCF-7 | Breast adenocarcinoma; PE, Tumorigenicity +, Erα +, E-Cadherin +, Vimentin- | 70.1 ± 14.5 |
| MDA-MB-453 | Metastatic breast carcinoma; PE, Tumorigenicity -, Erα-, E-Cadherin-, Vimentin- | 65.9 ± 20.2 |
| MDA-MB-435s | Metastatic ductal adenocarcinoma, Tumorigenicity +, metastatic, Erα-, E –Cadherin - , Vimentin + | 571.5 ± 18.7 |
| MDA-MB-231 | Breast adenocarcinoma: PE, Tumorigenicity +, metastatic, Erα-, E – Cadherin - , Vimentin + | 190.0 ± 9.6 |

**Table 1: Comparison of basal levels of VEGF secreted by cell lines representing models of earlier and later stages of breast cancer.** MCF-7, MDA-MB-453, MDA-MB-231, and MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. MCF-7 and MDA-MB-453 cells are models of earlier stages of breast cancer while MDA-MB-231 and MDA-MB-435s are models of later stages of breast cancer (6)
PE, Pleural Effusion; ERα, Estrogen Receptor α. Results represent four independent experiments ± SEM.

## Figures

**Figure 1: Structures of Chemically Modified Tetracyclines. A.** CMT 300, 6-demethyl-6-deoxy-4-dedimethyl-aminotetracycline **B.** CMT 308, 9-amino-6-demethyl-6-deoxy-4-dedimethyl-aminotetracycline

**Figure 2: Effects of TGFβ treatment on VEGF secretion by cell lines representing models of earlier and later stages of breast cancer.** MCF-7 (black bars), MDA-MB-453 (open bars), MDA-MB-231 (hatched bars) and MDA-MB-435s (crossed bars) cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ as indicated. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. TGFβ concentrations from 0.1 ng/ml to 10 ng/ml significantly stimulated VEGF secretion from MCF-7 cells ($p=0.0028$), MDA-MB-453 cells ($p=0.001$), MDA-MB-231 cells ($p<0.0001$) and MDA-MB-435s cells ($p=0.0006$). There was a significant difference between VEGF levels secreted by all the cells treated with 0 ng/ml TGFβ ($p=0.0018$), 0.1 ng/ml TGFβ ($p<0.0001$), 1 ng/ml TGFβ ($p=0.0005$) and 10 ng/ml TGFβ ($p<0.0001$). Results represent the mean of at least three independent experiments ± SEM.

**Figure 3: Effects of TGFβ and IGF-I on bFGF secretion by MCF-7 cells.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ (black bars) or IGF-I (open bars) as indicated. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for bFGF by ELISA. Neither TGFβ nor IGF-I significantly increased bFGF secretion from MCF-7 cells. Results represent mean of three independent experiments ± SEM.

**Figure 4: Effects of TGFβ and IGF-I on VEGF secretion by MCF-7 and MDA-MB-231cells. A.** MCF-7 or **B.** MDA-MB-231 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ (black bars) or IGF-I (open bars) as indicated. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. **A.** TGFβ significantly ($p<0.05$) increased VEGF secretion from MCF-7 cells, while IGF-I did not. Results represent mean of three independent experiments ± SEM. **B.** TGFβ and IGF-I significantly ($p<0.0001$) increased VEGF secretion from MDA-MB-231 cells. Results represent mean of four independent experiments ± SEM.

**Figure 5: CMT 308 inhibits basal and TGFβ-stimulated VEGF secretion from MDA-MB-453 cells.** MDA-MB-453 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ as indicated. In addition, the cells received either diluted DMSO vehicle alone (black bars), 5 μM CMT 300(clear bar), or 20 μM CMT 308 (hatched bar). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. CMT 308 significantly reduced VEGF secretion in cells treated with 0.1 and 10 ng/ml TGFβ relative to cells to which no tetracycline had been added. CMT 300 at 5 μM did not significantly affect VEGF secretion except in 10 ng/ml TGFβ treated cells. Results represent mean of three independent experiments ± SEM. (*$p<0.05$).

**Figure 6: CMT 308 inhibits basal and TGFβ-stimulated VEGF secretion from MCF-7 cells. A.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ as indicated. In addition, the cells received either diluted DMSO vehicle alone (black bars), 5 μM CMT 300

(clear bars), 20 μM CMT 300 (grey bars) or 20 μM CMT 308 (hatched bars). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. Doses of TGFβ from 0.1 ng/ml to 10 ng/ml significantly stimulated VEGF secretion from MCF-7 cells. CMT 308 significantly reduced VEGF secretion relative to cells treated with vehicle alone. CMT 300 at 5 μM did not significantly affect VEGF secretion, but at 20 μM significantly increased VEGF secretion. Results represent the mean ± SEM. n=3-6. (*$p<0.05$), (**$p<0.005$). **B.** Total Protein levels in conditioned medium MCF-7 cells treated with 0.1, 1 or 10 ng/ml TGFβ and either DMSO (black bars) or 5 μM CMT 300 (clear bars) or 20 μM CMT 308 (hatched bars). No significant change in total protein levels were observed in cells treated with TGFβ compared to untreated cells or in cells treated with either of the CMTs compared to untreated cells. Results represent mean of four independent experiments ± SEM.

**Figure 7: Effects of CMTs on VEGF secretion by MCF-7 cells treated with IGF-I.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml IGF-I as indicated. In addition, the cells received either diluted DMSO vehicle alone (black bars), 5 μM (clear bar) CMT 300 or 20 μM (hatched bar) CMT 308. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. IGF-I did not significantly stimulate VEGF secretion from MCF-7 cells, and CMT 300 did not significantly affect VEGF secretion. CMT 308 significantly ($p<0.005$) reduced VEGF secretion relative to mock-treated cells in the presence and absence of IGF-I. Results represent mean of four independent experiments ± SEM.

**Figure 8: CMTs are minimally cytotoxic to MCF-7 cells.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 5, 10, 20, or 30 μM CMT 300 or CMT 308 for 24h. Thereafter, medium was replaced with serum-free medium and MTS solution was added for up to 4 hours;, the absorbance of the soluble formazan product generated by viable cells was read at 490 nm. The viability of cells exposed to each CMT was calculated relative to untreated control cells. Neither CMT 300 in (-■-) nor CMT 308 (-□-) was cytotoxic to up to 30 μM in MCF-7 cells. Results represent mean of four independent experiments ± SEM.

**Figure 9: CMT 308 reduces TGFβ-induced VEGF secretion from MCF-7 cells in a dose-dependent manner.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with diluted DMSO vehicle alone or 1, 5, 20, 30 or 50 μM CMT 308 in addition to 1 ng/ml TGFβ as indicated. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. CMT 308 caused a significant ($p<0.0001$) dose-dependent reduction in VEGF secretion from MCF-7 cells. Results represent mean of six independent experiments ± SEM.

**Figure 10: Time course of the effect of CMTs on VEGF secretion by MCF-7 cells.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated either with diluted DMSO vehicle alone (black bars), with 20 μM CMT 308 alone (grey bar), with 10 ng/ml TGFβ alone (hatched bar), or with a combination of 20 μM CMT 308 and 10 ng/ml TGFβ (spotted bar). At specific times after treatment as indicated, conditioned medium was collected and assayed for VEGF by ELISA. Mock – treated cells released significant levels of VEGF over 24h ($p<0.0001$). Treatment with TGFβ alone over 24h significantly increased VEGF production ($p<0.0001$). CMT 308-treated cells did not release significant levels of VEGF up to 24h in the absence of TGFβ. CMT 308-treated cells maintained in the presence of TGFβ did not release significant levels of VEGF up to 8h; thereafter these

cells began to secrete VEGF in the presence of CMT 308. VEGF levels were significant by 24h ($p<0.05$). Results represent mean of three independent experiments ± SEM.

**Figure 11: CMT 308 is effective for up to 8 hours of treatment in MCF-7 cells regardless of time before initiation of treatment.** MCF-7 cells were plated in complete medium and incubated overnight at $37^0$C. Next day, medium was replaced with serum-free medium. One set of cells was left untreated (black bars) and conditioned medium was collected from these cells immediately after plating at 0h (Bar A) or 8h after plating (Bar B) or 16h after plating (Bar C). A second set of cells was treated with 20 μM CMT 308 at the time of plating at 0h (clear bars). Conditioned medium was collected from these cells immediately upon plating (Bar D), 8h after plating (Bar E) or 16h after plating (Bar F). A third set of cells was treated with CMT 308 8h after plating (hatched bars). Conditioned media from these cells was collected 8h after plating, that is, 0h after adding CMT 308 (Bar G) or 16h after plating, that is, 8h after adding CMT 308 (Bar H). Results represent mean of three independent experiments ± SEM. (***$p<0.0001$)

**Figure 12: TGFβ stimulated pathways are sensitive to CMT 308 mediated inhibition.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium, and incubated further for either 22h (bar 1) or 30h (bar 4) without additions. Another set of cells were exposed for 16h (bar 2) or 24h (bar 5) in serum-free medium containing 20 μM CMT 308 followed, without change of medium, by an additional 6h in sham. Another set of cells were treated for 16h (bar 3) or 24h (bar 6) in serum-free medium containing 20 μM CMT 308 followed by addition of 10 ng/ml TGFβ without replacement of the original medium. Incubation was then continued for an additional 6 hours. Cells maintained for 6h in the presence of TGFβ after 24h in CMT 308 alone secreted significantly ($p<0.05$) higher levels of VEGF (60 pg/ml) within the 6h interval than cells maintained for 6h in the presence of TGFβ after 16h in CMT 308 (23 pg/ml). All values in the presence of CMT 308 were consistently lower than those in the presence of sham. Results represent mean of three independent experiments ± SEM.

**Figure 13: Sub-cytotoxic doses of CMT 308 do not reduce VEGF mRNA levels in MCF-7 cells.** MCF-7 cells were treated with 1 ng/ml TGFβ and a range of doses of CMT 308 for 24h. Equal quantities of total RNA were assayed using PCR. VEGF primers were chosen to distinguish between the mRNAs for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, and $VEGF_{121}$ isoforms. The only VEGF mRNA species detectable were for the $VEGF_{165}$ and $VEGF_{121}$ isoforms. TGFβ did not increase VEGF mRNA levels (Lanes 1 and 2). Treatment with CMT 308 from 1 μM to 50 μM (Lanes 3-7) did not decrease VEGF mRNA levels in MCF-7 cells. Results represent three independent experiments.

**Figure 14: TGFβ does not increase VEGF mRNA and CMT 308 does not reduce VEGF mRNA levels in MCF-7 cells.** MCF-7 cells were treated with 5 μM CMT 300 or 20 μM CMT 308 in the absence (Lanes 1-3) or presence (Lanes 4-6) of 1 ng/ml TGFβ for 6h. **A.** Equal quantities of total RNA were assayed using PCR. The only VEGF mRNA species detectable were for the $VEGF_{165}$ and $VEGF_{121}$ isoforms. Treatment with TGFβ alone did not cause an increase in VEGF mRNA levels (Lanes 1 & 4). Neither addition of CMT 300 nor CMT 308 reduced VEGF mRNA in cells cultured in the presence (Lanes 5 & 6) or absence of TGFβ (Lanes 2 & 3). Results represent three independent experiments.

**B.** Quantitation of VEGF mRNA levels relative to GAPDH mRNA levels by quantitative hybridization to an immobilized consensus complementary oligonucleotide sequence. VEGF mRNA levels were not significantly reduced in MCF-7 cells treated with 20 μM CMT 308

(hatched bars) relative to mock-treated (black bars) cells in TGFβ-treated or untreated cells. Results represent mean of three independent experiments ± SEM.

**Figure 15: CMT 308 inhibits basal and TGFβ-stimulated VEGF secretion from MDA-MB-231 cells.** MDA-MB-231 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ as indicated. In addition, the cells received either diluted DMSO vehicle alone (black bar), 5 μM CMT 300 (open bar), or 20 μM CMT 308 (hatched bar). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. TGFβ at 0.1, 1 and 10 ng/ml significantly (***$p<0.0001$) stimulated VEGF secretion over the levels released by unstimulated MDA-MB-231 cells. CMT 308 significantly decreased basal (*$p=0.0032$) and TGFβ-stimulated VEGF secretion at concentrations of growth factor of 0.1 ng/ml (**$p=0.0016$), 1 ng/ml (**$p=0.0005$), and 10 ng/ml (*$p=0.0056$). CMT 300 at 5 μM did not significantly reduce VEGF secretion. Results represent mean of four independent experiments ± SEM.

**Figure 16: CMT 308 inhibits basal and TGFβ-stimulated VEGF secretion from MDA-MB-435s cells.** MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 0.1, 1 or 10 ng/ml TGFβ as indicated. In addition, the cells received either diluted DMSO vehicle alone (black bar), 5 μM CMT 300 (open bar) CMT 300, 20 μM CMT 300 (grey bar), or 20 μM CMT 308 (hatched bar). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. TGFβ at 1 and 10 ng/ml significantly (***$p<0.0001$) stimulated VEGF secretion from MDA-MB-435s cells. CMT 308 significantly decreased basal (***$p<0.0001$) and TGFβ-stimulated VEGF secretion at levels of growth factor of 0.1 ng/ml and 1 ng/ml (***$p<0.0001$) as well as at 10 ng/ml (**$p<0.01$). CMT 300 at 5 μM did not affect VEGF secretion significantly, but in the presence of 20 μM CMT 300, VEGF secretion was significantly increased, either in the absence of TGFβ (***$p<0.0001$) or in the added presence of 0.1 ng/ml TGFβ (**$p<0.001$). In the presence of 20 μM CMT 300 and 1 ng/ml TGFβ, VEGF secretion was significantly decreased (*$p<0.01$) compared to untreated cells. Results represent mean of six independent experiments ± SEM.

**Figure 17: CMTs are minimally cytotoxic to MDA-MB-435s and MDA-MB-231 cells.** MDA-MB-435s and MDA-MB-231 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with various concentrations of CMT 300 or CMT 308 from 1 μM to 100 μM for 24h. Thereafter, medium was replaced with serum-free medium and MTS solution added for up to 4 hours, and absorbance of the soluble formazan product generated by viable cells read at 490 nm. The numbers of viable cells in the presence of the tetracycline derivatives were calculated relative to control cells cultured in the absence of CMTs. Neither CMT 300 (-■-) nor CMT 308 (-□-) was significantly cytotoxic to MDA-MB-435s cells at concentrations up to 100 μM. CMT 300 (-▲-) at concentrations up to 10 μM and CMT 308 (-△-) at concentrations up to 30 μM were not significantly cytotoxic to MDA-MB-231 cells. Results represent mean of six independent experiments ± SEM.

**Figure 18: CMT 308 reduces basal VEGF secretion from MDA-MB-435s cells in a dose-dependent manner.** MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and 1, 5, 10, 20, 30 or 50 μM CMT 300 (black bars) or CMT 308 (clear bars) was added. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. CMT 308 at 20, 30 and 50 μM

significantly reduced VEGF secretion. CMT 300 from 5 to 50 μM significantly increased VEGF secretion compared to untreated cells, at levels of significance as indicated (*p<0.05, **p<0.005). Results represent means of six independent experiments ± SEM.

**Figure 19: TGFβ does not increase VEGF mRNA and CMT 308 does not reduce VEGF mRNA levels in MDA-MB-435s cells.** MDA-MB-435s cells were cultured either in the absence (Lanes 1-3) or presence (Lanes 4-6) of TGFβ and were treated with 5 μM CMT 300 or 20 μM CMT 308 for 6h. Equal quantities of total RNA were assayed using PCR. The only VEGF mRNA species detectable were for the $VEGF_{165}$ and $VEGF_{121}$ isoforms. Treatment with 1 ng/ml TGFβ did not cause an increase in VEGF mRNA levels (Lanes 1 & 4). Neither CMT 300 (5 μM) nor CMT 308 (20 μM) reduced VEGF mRNA in cells cultured in the presence (Lanes 5 & 6) or the absence of TGFβ (Lanes 2 & 3). Results are representative of three independent experiments.

**Figure 20: TGFβ increases intracellular VEGF levels and CMT 308 reduces intracellular VEGF levels in MDA-MB-435s cells.** MDA-MB-435s cells were cultured either in the presence or the absence of 1 ng/ml TGFβ as indicated. Cells were also either sham-treated or treated with 20 μM CMT 300 or 20 μM CMT 308 for 24h as indicated. Conditioned media and cell lysates were collected and analyzed for VEGF by ELISA. VEGF levels shown have been corrected for total volume of cell lysates (100 μL) and conditioned media (10 ml). Total VEGF levels in cell lysates (black bars) are represented on the Left Y axis, while the levels of VEGF in conditioned media (clear bars) are represented on the Right Y axis. Results are representative of two independent experiments.

**Figure 21: Cytotoxicity of CMTs on MonoMac 6 cells.** MonoMac 6 cells were plated in serum-free medium and treated with 5, 10 or 20 μM CMT 300 (-■-) or CMT 308 (-▲-) for 24h. After CMT-treatment MTS solution was added to cells for up to 4 hours, and absorbance of viable cells was read at 490 nm. Cytotoxicity in the presence of 20 μM CMT 300 (-■-) was significant (*p<0.05) while in the presence of CMT 308 (-▲-) it was not significant. Results represent mean of three independent experiments ± SEM.

**Figure 22: Dose Response of CMTs on MonoMac 6 cells.** MonoMac 6 cells were plated in serum-free medium and treated with 5, 10 or 20 μM CMT 300 (black bars) or CMT 308 (clear bars) for 24h. **A.** Conditioned medium was assayed for VEGF by ELISA. CMT 308 at 20 μM very significantly (***p<0.0001) reduced VEGF secretion compared to mock-treated cells while 20 μM CMT 300 also reduced VEGF, but at a lower level of significance (*p<0.05) in these cells. **B.** Total protein levels in conditioned media were not affected significantly by either CMT 300 or CMT 308. Results represent mean of three independent experiments ± SEM.

**Figure 23: CMTs do not reduce IL-8 secretion from MonoMac 6 cells.** MonoMac 6 cells were plated in serum-free medium and treated with either 5μM CMT 300 or 20 μM CMT 308 for 24h. Conditioned medium was then assayed for IL-8 by ELISA. Neither CMT 308 nor CMT 300 significantly reduced IL-8 secretion from MonoMac 6 cells. Results represent mean of three independent experiments ± SEM.

**Figure 24: Time course of VEGF secretion by MonoMac 6 cells.** MonoMac 6 cells were plated in serum-free medium and cells were either sham-treated (black bars) or treated with 20 μM CMT 308 (clear bars). At specific times after treatment, conditioned medium was removed and assayed for VEGF by ELISA. In the absence of the tetracycline derivative, MonoMac 6 cells secreted significant levels of VEGF which could be detected within 4h of plating (*p<0.05) and even more significant amounts (**p<0.001) 8h after plating. Treatment with CMT 308

significantly reduced (*p<0.005) VEGF secretion at 8h; this diminution was still significant at 24h. Results represent mean of three independent experiments ± SEM.

**Figure 25: TGFβ does not stimulate VEGF secretion and CMT 308 inhibits basal VEGF secretion from MonoMac 6 cells.** MonoMac 6 cells were plated in serum-free medium and treated with 0.1, 1 or 10 ng/ml of TGFβ. They then received sham-treatment (black bars), 5 μM CMT 300 (clear bar), or 20 μM CMT 308 (hatched bar). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was collected and assayed for VEGF by ELISA. TGFβ did not stimulate VEGF secretion. CMT 308 significantly (***p<0.0001) reduced VEGF secretion in cells regardless of TGFβ treatment. CMT 300 did not significantly affect VEGF secretion. Results represent mean of three independent experiments ± SEM.

**Figure 26: VEGF secretion by MonoMac 6 cells cultured in serum is not inhibited by CMTs.** MonoMac 6 cells were plated in complete medium containing 10% FBS in the presence of 0, 5, 10 or 20 μM CMT 300 (black bars) or CMT 308 (clear bars) and incubated for 24h at $37^0$C. Conditioned medium was collected and assayed for VEGF by ELISA. No significant differences were observed among the VEGF levels secreted by cells cultured in serum-containing medium alone or medium to which either of the CMTs had been added. Results represent mean of four independent experiments ± SEM.

**Figure 27: CMT 308 does not significantly reduce intracellular VEGF levels in MonoMac 6 cells up to 24h. A.** MonoMac 6 cells were cultured either in serum-free medium alone (Lanes 1, 6), or in medium containing 5 μM CMT 300 (Lanes 2, 7) or 20 μM CMT 308 (Lanes 3, 8). Equal quantities of protein from cell lysates (Lanes 1-3) or conditioned media (Lanes 6-8) were analyzed by western blotting using a monoclonal antibody to VEGF. Lanes 4 and 5 are blank. Results represent three independent experiments. **B.** Total intracellular protein levels did not change significantly after culture of the cells in the presence of the CMTs. Results represent mean of two independent experiments ± SEM.

**Figure 28: CMT 308 reduces VEGF mRNA levels in MonoMac 6 cells. A.** MonoMac 6 cells were cultured in serum-free medium alone (Lane 1) or in medium containing 5 μM CMT 300 (Lane 2) or 20 μM CMT 308 (Lane 3) for 6h. Equal quantities of total RNA were assayed using PCR. The only VEGF mRNA species detectable were for the $VEGF_{165}$ and $VEGF_{121}$ isoforms. Results are representative of three independent experiments. **B.** MonoMac 6 cells were cultured in serum-free medium alone or in medium containing 10 μM CMT 308 for 6h. VEGF mRNA levels were quantitated relative to GAPDH mRNA levels in cell lysates by hybridization to a consensus complementary oligonucleotide immobilized on a multiwell microplate using the Quantikine mRNA assay. CMT 308 (10 μM) significantly (p<0.05) reduced VEGF mRNA levels relative to GAPDH mRNA levels. Results represent mean of three independent experiments ± SEM.

**Figure 29: Cytotoxicity of CMTs on HUVEC.** HUVEC ($4 \times 10^5$ cells/ml) in complete medium were plated on 96-well plates and treated with DMSO vehicle alone or various concentrations of CMT 300 (-■-) or CMT 308 (-▲-) for 24h. After CMT-treatment MTS solution was added to cells for up to 4 hours, and absorbance of viable cells was read at 490 nm. Cytotoxicity was significant (p=0.0002) only in the presence of higher doses (100 μM) of CMT 300, whereas cytotoxicity over the entire dose range of CMT 308 never reached a significant level. Results represent mean of three independent experiments ± SEM.

**Figure 30: Effect of CMT 300 on tubular network formation by HMVEC on Matrigel.** HMVEC ($4 \times 10^5$ cells/ml) in complete medium were treated with various concentrations of

CMT 300 in a microfuge tube and immediately plated onto a 96-well plate coated with a thick layer of Matrigel at a density of $2x10^4$ cells/well for 18h. Wells were then washed with HBSS and the cells were stained with Calcein AM for 45min at $37^0$C. After washing off excess dye, tube formation by viable cells was observed under a fluorescent microscope at 4x magnification. **A.** Sham-treated, **B.** 1 µM CMT 300, **C.** 5 µM, **D.** 10 µM, **E.** 20 µM, **F.** 30 µM, **G.** 50 µM. Results are representative of four independent experiments.

**Figure 31: Effect of CMT 308 on tubular network formation by HMVEC on Matrigel.** HMVEC ($4 \times 10^5$ cells/ml) in complete medium were treated with various concentrations of CMT 308 in a microfuge tube, and then immediately plated onto a 96-well plate coated with a thick layer of Matrigel at a density of $2x10^4$ cells/well for 18h. Wells were then washed with HBSS and the cells were stained with Calcein AM for 45min at $37^0$C. After washing off excess dye, tube formation by viable cells was observed under a fluorescent microscope at 4x magnification. **A.** Sham-treated, **B.** 1 µM CMT 308, **C.** 5 µM, **D.** 10 µM, **E.** 20 µM, **F.** 30 µM, **G.** 50 µM. Results are representative of four independent experiments.

**Figure 32: Cytotoxicity of CMTs to HMVEC plated on Matrigel.** HMVEC ($4x10^5$ cells/ml) in complete medium were plated onto 96-well plates coated with matrigel and treated with DMSO or various concentrations of CMT 300 (-■-) or CMT 308 (-▲-) for 22h. After this 22 h incubation, MTS solution was added to cells for up to 4 hours, and absorbance of the formazan generated by viable cells was read at 490 nm. Cytotoxicity was significant ($p<0.05$) in the presence of 1 µM CMT 308 while in the presence of CMT 300 it was not significant. Results represent mean of three independent experiments ± SEM.

**Figure 33: Effect of CMT 308 on reticular network formation by HMVEC on Collagen I.** HMVEC ($4 \times 10^5$ cells/ml) in complete medium were treated with various concentrations of CMT 308 in a microfuge tube, and then immediately plated onto a 96-well plate coated with type I collagen at a density of $2x10^4$ cells/well for 18h. Tubular network formation was observed under a phase contrast microscope at 4x magnification. **A.** Sham-treated, **B.** 30 µM CMT 308. Results are representative of three independent experiments.

**Figure 34: Effect of CMT 300 on pre-formed reticular networks of HMVEC on collagen I.** $2x10^4$ HMVEC cells/well were plated in a 96-well plate pre-coated with Collagen I in complete medium. Cells were incubated for 22h at $37^0$C. Reticular network formation was confirmed by observing under a phase contrast microscope. Cells were then treated with DMSO or 30 µM CMT 300 for a further 18h. The morphology of the pre-formed networks was observed under a phase contrast microscope at 4x magnification. **A.** Sham-treated, **B.** 30 µM CMT 300. Results are representative of three independent experiments.

**Figure 35: Effect of CMT 300 on migration of HUVEC through fibronectin coated inserts:** $1 \times 10^5$ cells/well were seeded on fibronectin-coated porous membrane-bottomed inserts for 24 well plates. Serum-free medium containing 0, 0.5, 2, 10 or 20 ng/ml VEGF was added to the surrounding wells only, and 10 µM CMT 300 was added to the inserts and the surrounding wells. The assembled plates were then incubated for 22h at $37^0$C. The medium in the surrounding wells was replaced with fresh medium containing Calcein AM for 90 min at $37^0$C. Fluorescence of viable cells on the bottom face of the inserts in the assembled plates was read on a Cytofluor 2350 microplate spectrofluorimeter set to 485 nm ex/530 nm em, and normalized to the fluorescence from wells without VEGF. Addition of 20 ng/ml VEGF to the surrounding wells significantly increased migration of cells ($p<0.05$) relative to sham treatment. CMT 300 did not

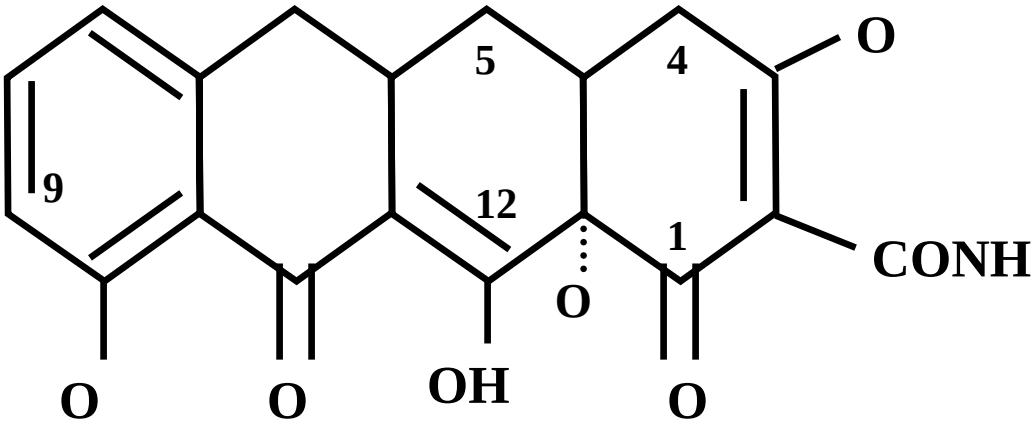
significantly reduce migration in cells. Results represent mean of three independent experiments ± SEM.

**Figure 36: Effect of CMT 308 on migration of HUVEC through fibronectin coated inserts:** 1 x $10^5$ cells/well were seeded on fibronectin-coated porous membrane-bottomed inserts for 96 well plates. Serum-free medium containing 0, 0.5, 2, 10 or 20 ng/ml VEGF was added to the surrounding wells only, and either DMSO (black bars) or 20 µM CMT 308 (hatched bars) was added to the inserts and the surrounding wells. The assembled plates were then incubated for 22h at $37^0$C. The medium in the surrounding wells was replaced with fresh medium containing Calcein AM for 90 min at $37^0$C. Fluorescence of viable cells on the bottom face of the inserts in the assembled plates was read on a Cytofluor 2350 microplate spectrofluorimeter set to 485 nm ex/530 nm em, and normalized to the fluorescence from wells without VEGF. 20 ng/ml VEGF significantly increased migration of cells ($p<0.05$) relative to sham treatment. CMT 308 significantly ($p<0.05$) reduced migration in cells treated with 20 ng/ml VEGF. Results represent mean of three independent experiments ± SEM.

**Figure 37: Effect of CMTs on invasion of HMVEC through Matrigel coated inserts:** 1 x $10^5$ cells/well were seeded on Matrigel-coated porous membrane-bottomed inserts for 24 well plates. SFM containing 0 (black bars), or 10 ng/ml (hatched bars) VEGF was added to the surrounding wells alone, and either DMSO, 20 µM 300 or 20 µM CMT 308 was added to the inserts and the surrounding wells. The assembled plates were then incubated for 22h at $37^0$C. The medium in the surrounding wells was replaced with fresh medium containing Calcein AM for 90 min at $37^0$C. Fluorescence of viable cells on the bottom face of the inserts in the assembled plates was read on a Cytofluor 2350 microplate spectrofluorimeter set to 485 nm ex/530 nm em, and normalized to the fluorescence from sham-treated cells in the presence or absence of VEGF added as chemoattractant. CMT 300 decreased invasion by 48% while CMT 308 reduced invasion by 73%. Results represent means of two independent experiments ± SEM, but the observed differences failed to meet the ANOVA test of significance.

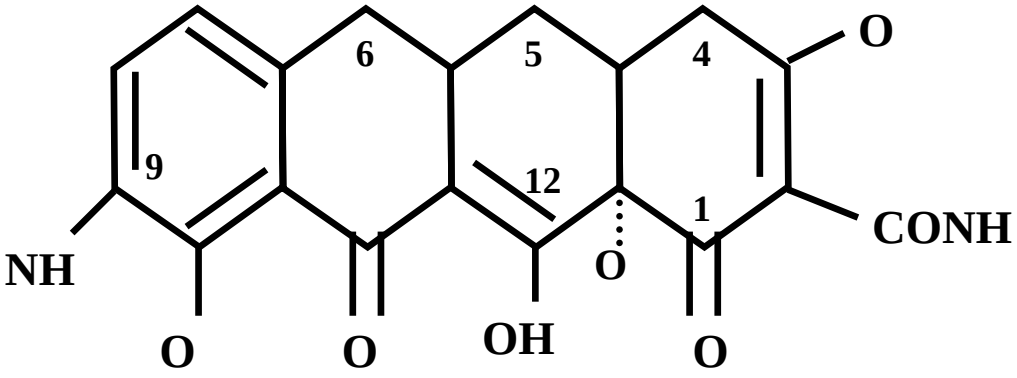
**Figure 38: Effect of conditioned media from CMT-treated MonoMac 6 cells on invasion of HMVEC through Matrigel coated inserts:** 1 x $10^5$ cells/well were seeded on Matrigel-coated porous membrane-bottomed inserts for 24 well plates. Conditioned medium from MonoMac 6 cells treated with either DMSO vehicle, 5 µM CMT 300 or 20 µM CMT 308 was added to the surrounding wells only. The assembled plates were then incubated for 22h at $37^0$C. The medium in the surrounding wells was replaced with fresh medium containing Calcein AM for 90 min at $37^0$C. Fluorescence of viable cells on the bottom face of the inserts in the assembled plates was read on a Cytofluor 2350 microplate spectrofluorimeter set to 485 nm ex/530 nm em, and normalized to the fluorescence from wells containing conditioned medium from vehicle-treated MonoMac 6 cells. Conditioned media from CMT 300-treated MonoMac 6 cells significantly ($p<0.05$) decreased invasion by HMVEC while conditioned media from CMT 308 treated cells did not. Results represent mean of three independent experiments ± SEM.

**Figure 1**

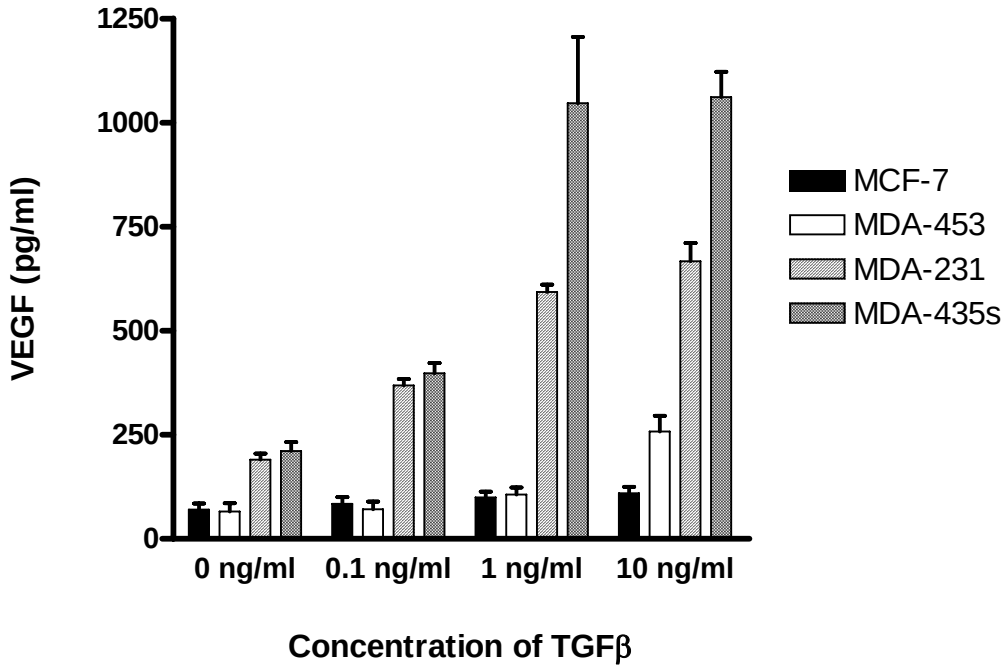



**A.**

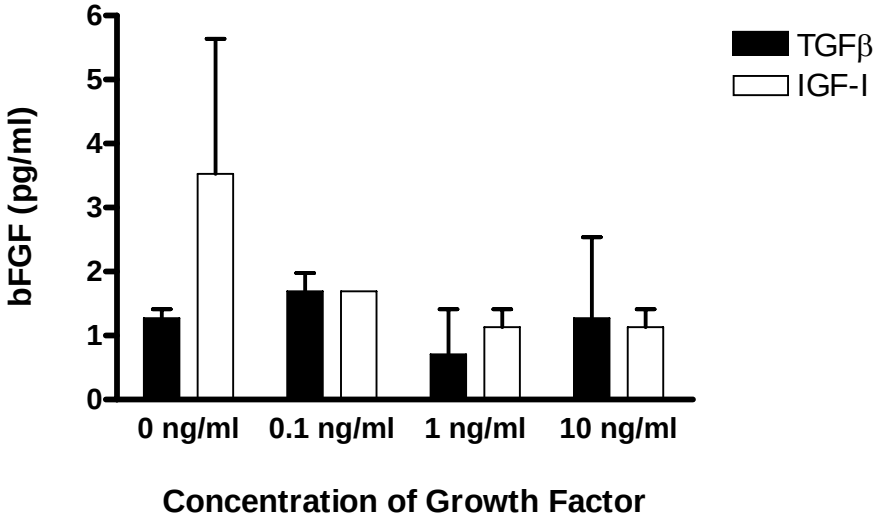
**B.**



# Figure 2

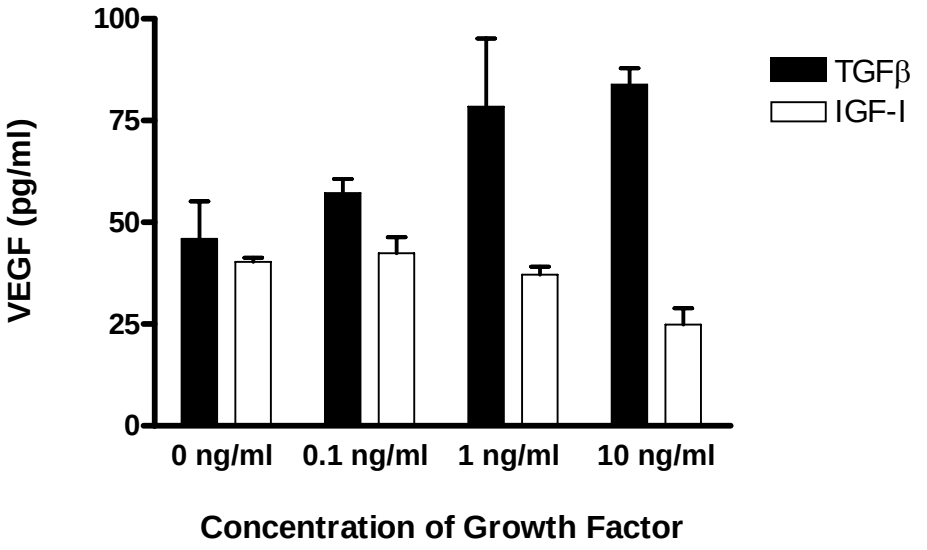

1250
1000
750
500
250
0
VEGF (pg/ml)
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
Concentration of TGFβ
MCF-7
MDA-453
MDA-231
MDA-435s

# Figure 3

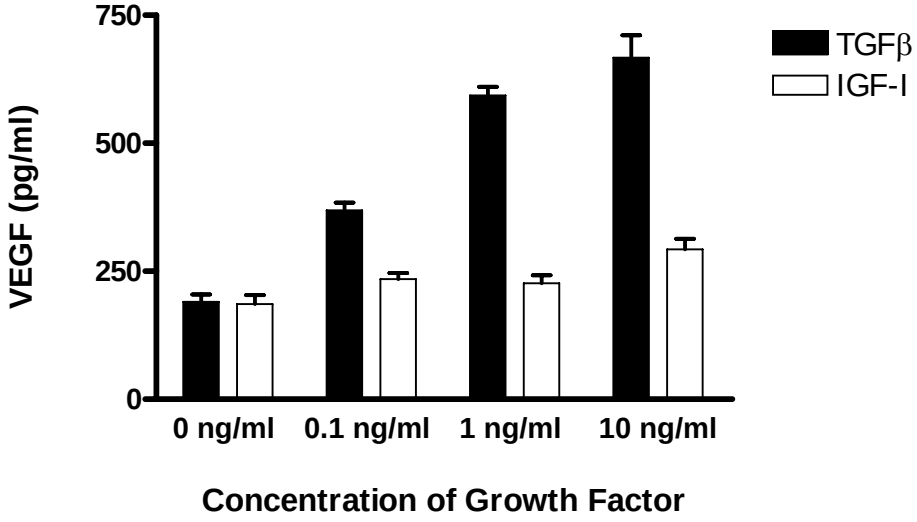

6
5
4
3
2
1
0
bFGF (pg/ml)
TGFβ
IGF-I
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
Concentration of Growth Factor

# Figure 4

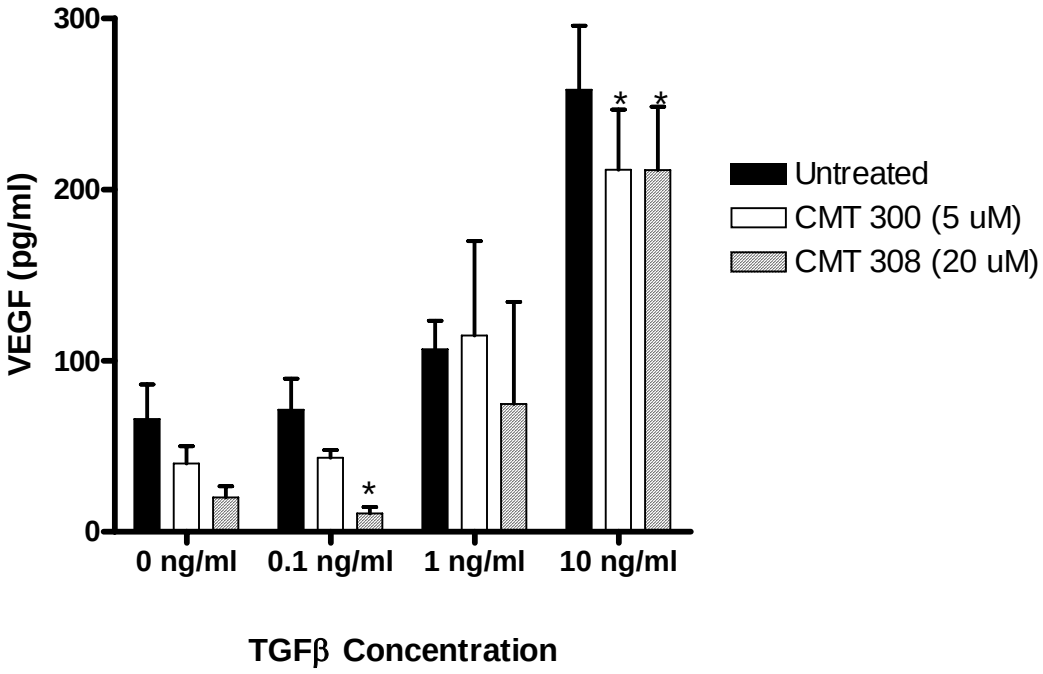
**A.**


100
75
50
25
0
VEGF (pg/ml)
TGFβ
IGF-I
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
Concentration of Growth Factor

**B.**

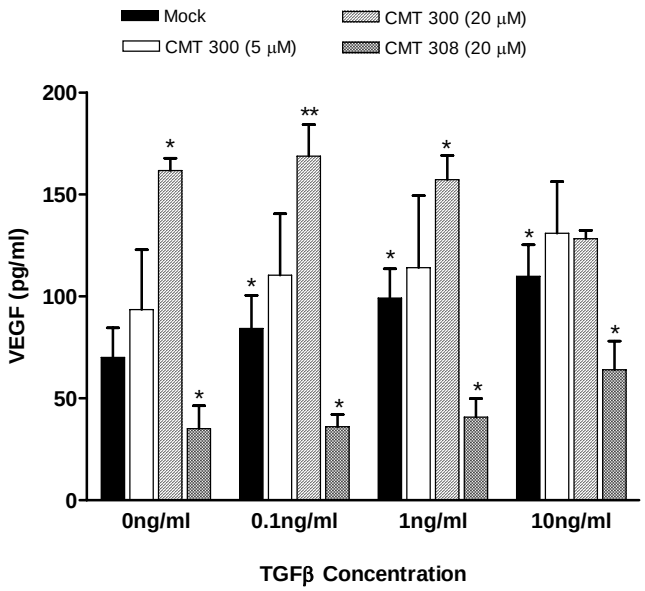

750
500
250
0
VEGF (pg/ml)
TGFβ
IGF-I
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
Concentration of Growth Factor

# Figure 5

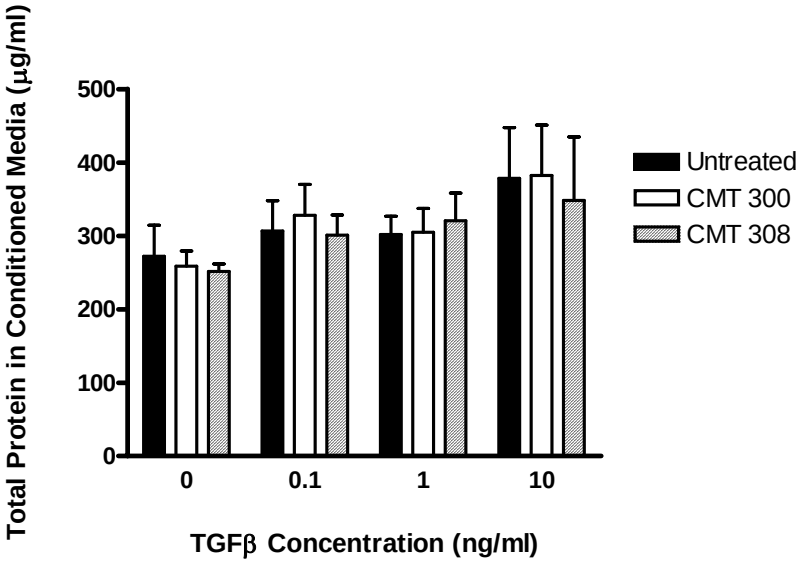

300
200
100
0
VEGF (pg/ml)
*
*
*
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ Concentration
Untreated
CMT 300 (5 uM)
CMT 308 (20 uM)

# Figure 6

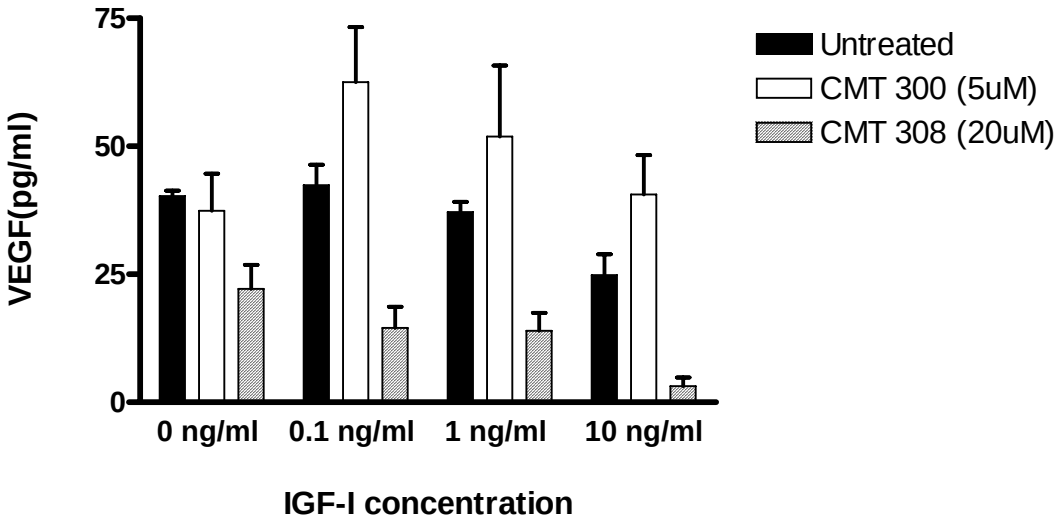
**A.**


Mock
CMT 300 (20 μM)
CMT 300 (5 μM)
CMT 308 (20 μM)
VEGF (pg/ml)
200
150
100
50
0
0ng/ml
0.1ng/ml
1ng/ml
10ng/ml
TGFβ Concentration

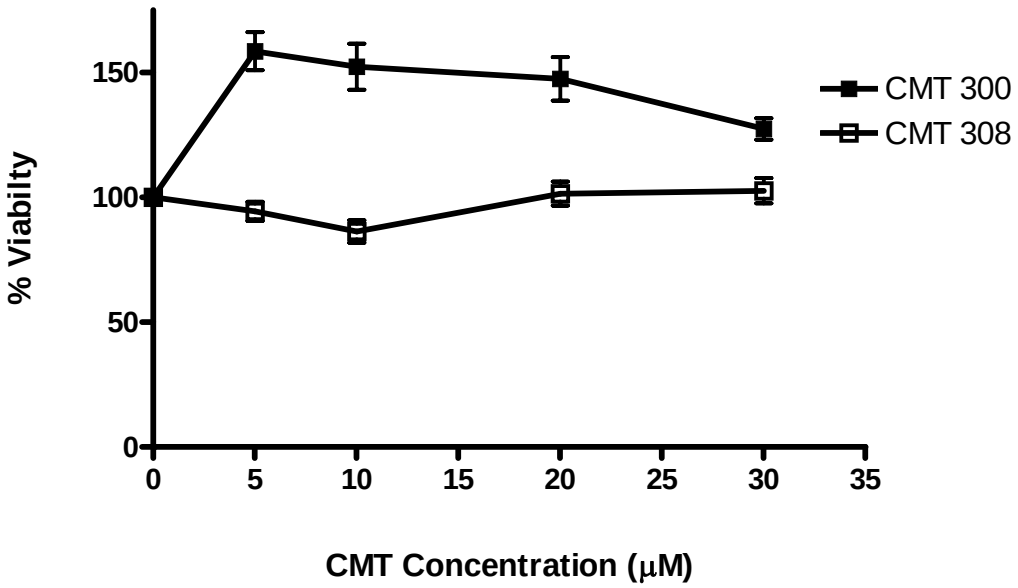
**B.**


Total Protein in Conditioned Media (μg/ml)
500
400
300
200
100
0
0
0.1
1
10
TGFβ Concentration (ng/ml)
Untreated
CMT 300
CMT 308

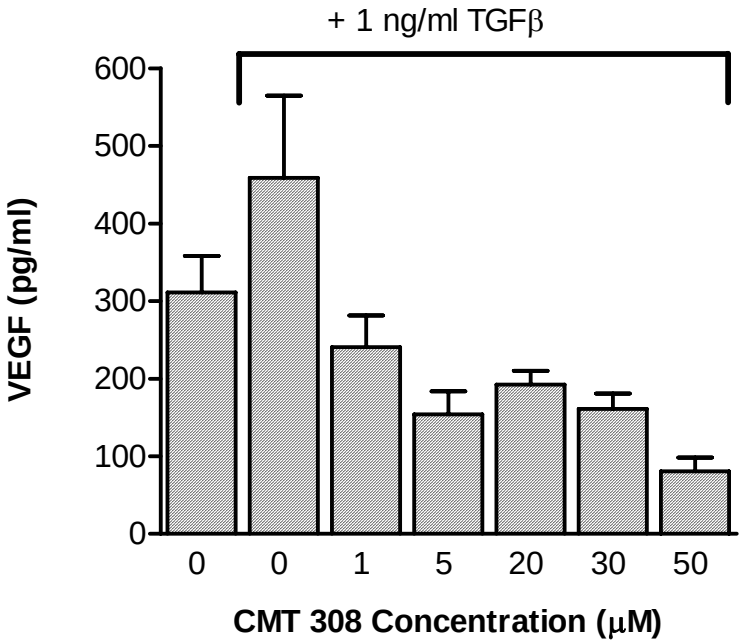
# Figure 7


75
50
25
0
VEGF(pg/ml)
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
IGF-I concentration
Untreated
CMT 300 (5uM)
CMT 308 (20uM)

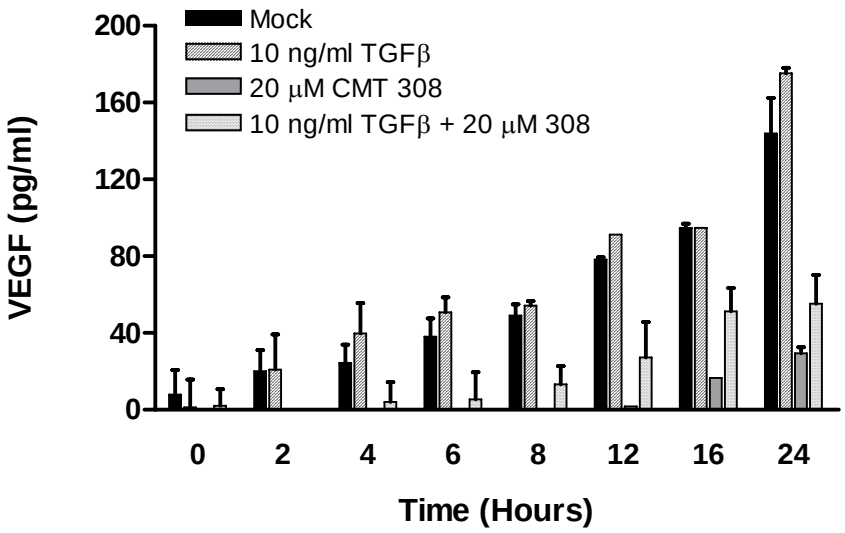
# Figure 8


% Viability
150
100
50
0
0
5
10
15
20
25
30
35
CMT Concentration (μM)
CMT 300
CMT 308

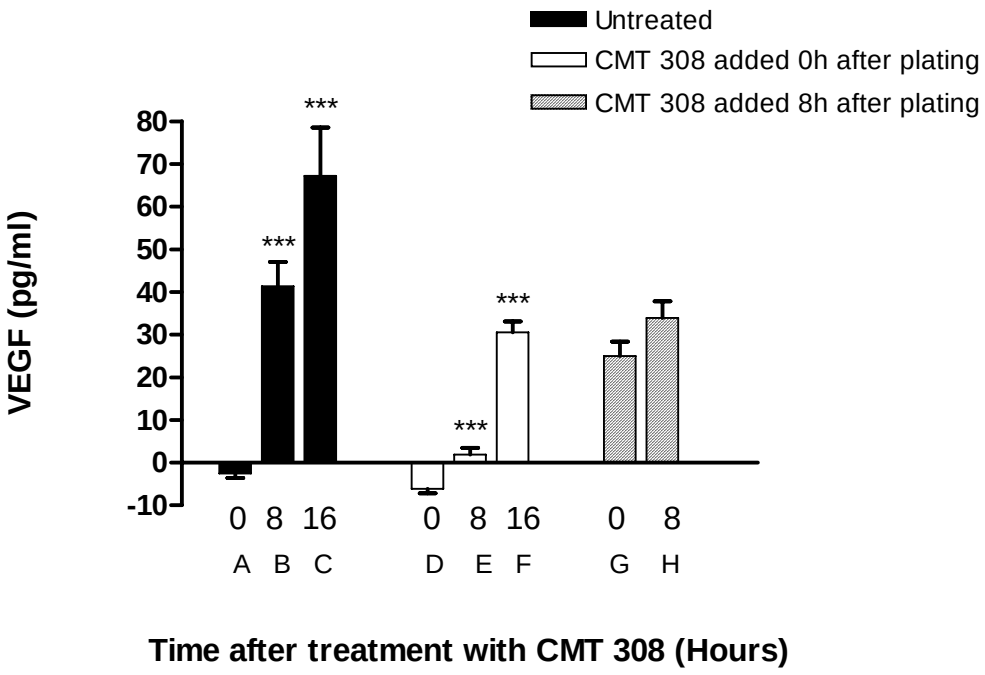
# Figure 9


+ 1 ng/ml TGFβ
600
500
400
300
200
100
0
VEGF (pg/ml)
0
0
1
5
20
30
50
CMT 308 Concentration (μM)

# Figure 10


Mock
10 ng/ml TGFβ
20 μM CMT 308
10 ng/ml TGFβ + 20 μM 308
VEGF (pg/ml)
200
160
120
80
40
0
0
2
4
6
8
12
16
24
Time (Hours)

# Figure 11


Untreated
CMT 308 added 0h after plating
CMT 308 added 8h after plating
VEGF (pg/ml)
80
70
60
50
40
30
20
10
0
-10
***
***
***
***
0 8 16 0 8 16 0 8
A B C D E F G H
Time after treatment with CMT 308 (Hours)

# Figure 12

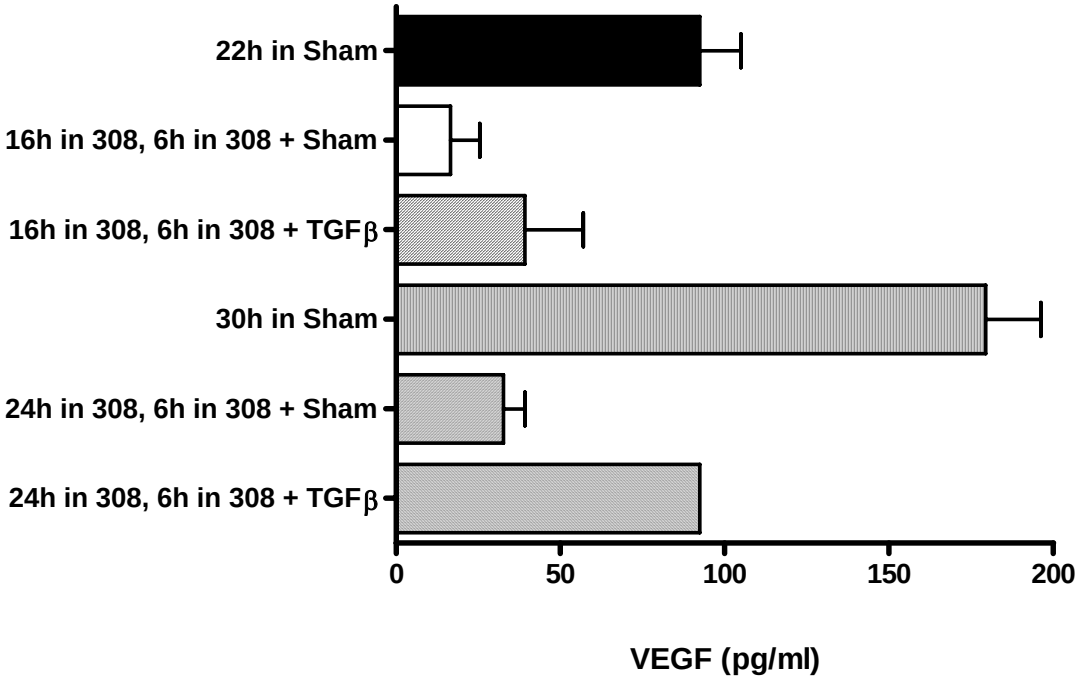
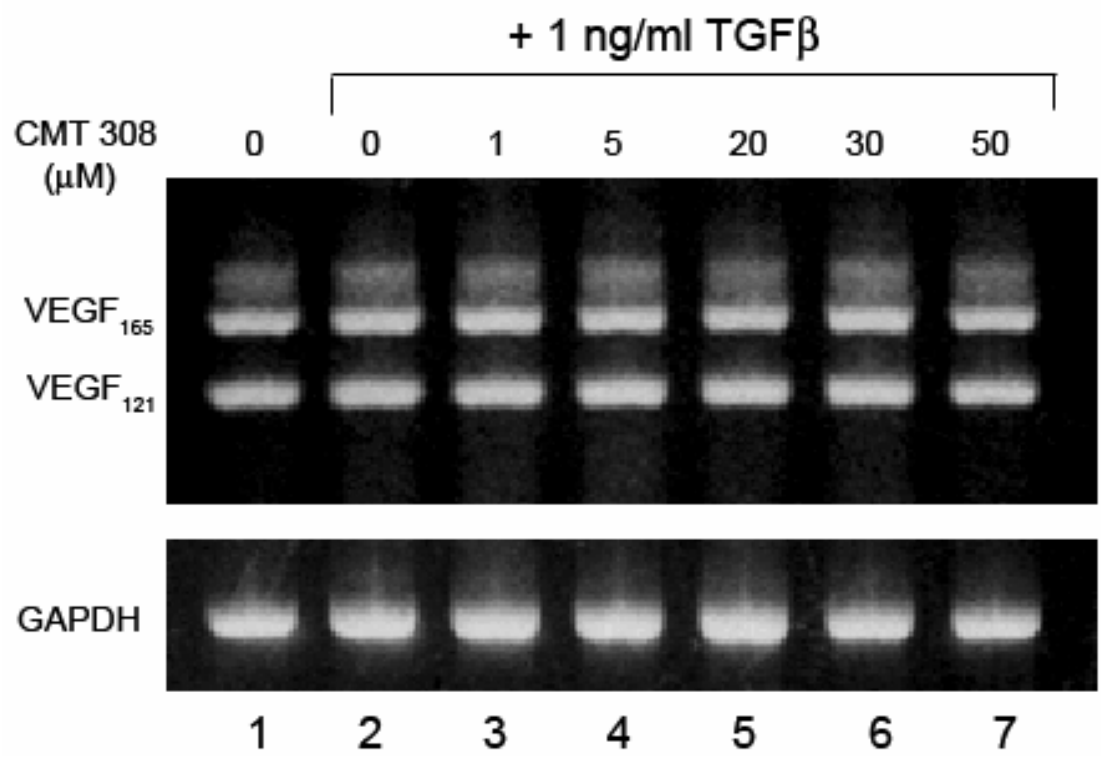
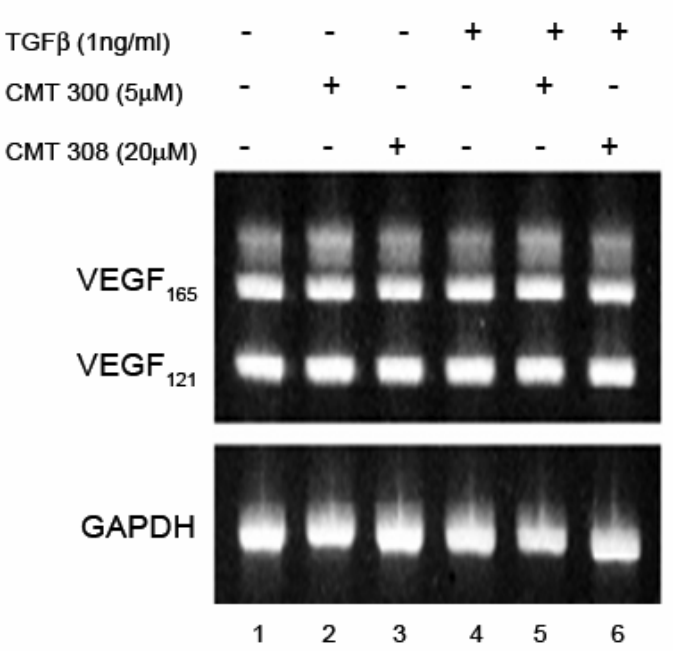

22h in Sham
16h in 308, 6h in 308 + Sham
16h in 308, 6h in 308 + TGFβ
30h in Sham
24h in 308, 6h in 308 + Sham
24h in 308, 6h in 308 + TGFβ
0
50
100
150
200
VEGF (pg/ml)

**Figure 13**

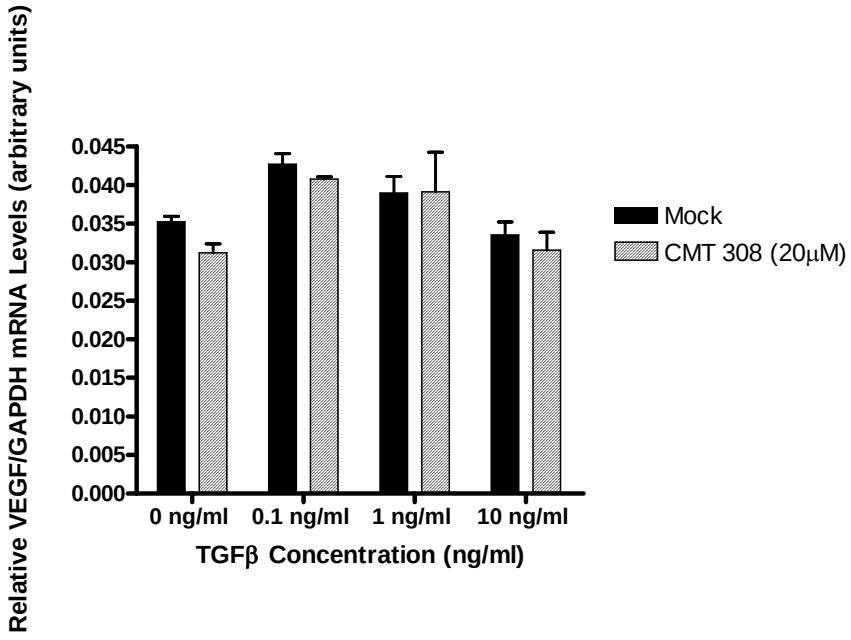

+ 1 ng/ml TGFβ
CMT 308 (μM)
0
0
1
5
20
30
50
VEGF165
VEGF121
GAPDH
1
2
3
4
5
6
7

# Figure 14

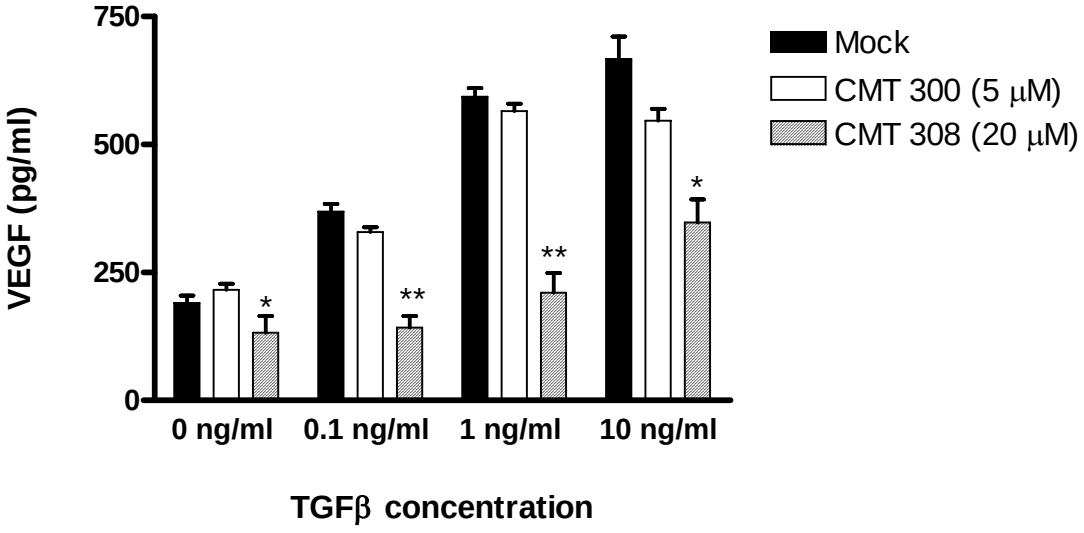
A.


TGFβ (1ng/ml) - - - + + +
CMT 300 (5μM) - + - - + -
CMT 308 (20μM) - - + - - +
VEGF165
VEGF121
GAPDH
1 2 3 4 5 6

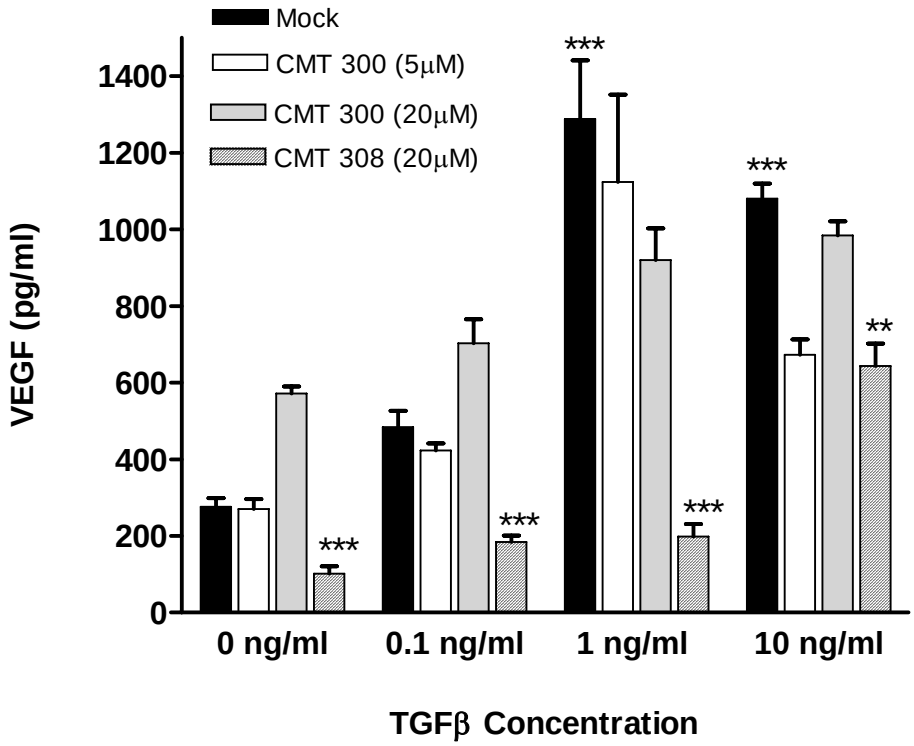
B.


Relative VEGF/GAPDH mRNA Levels (arbitrary units)
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ Concentration (ng/ml)
Mock
CMT 308 (20μM)

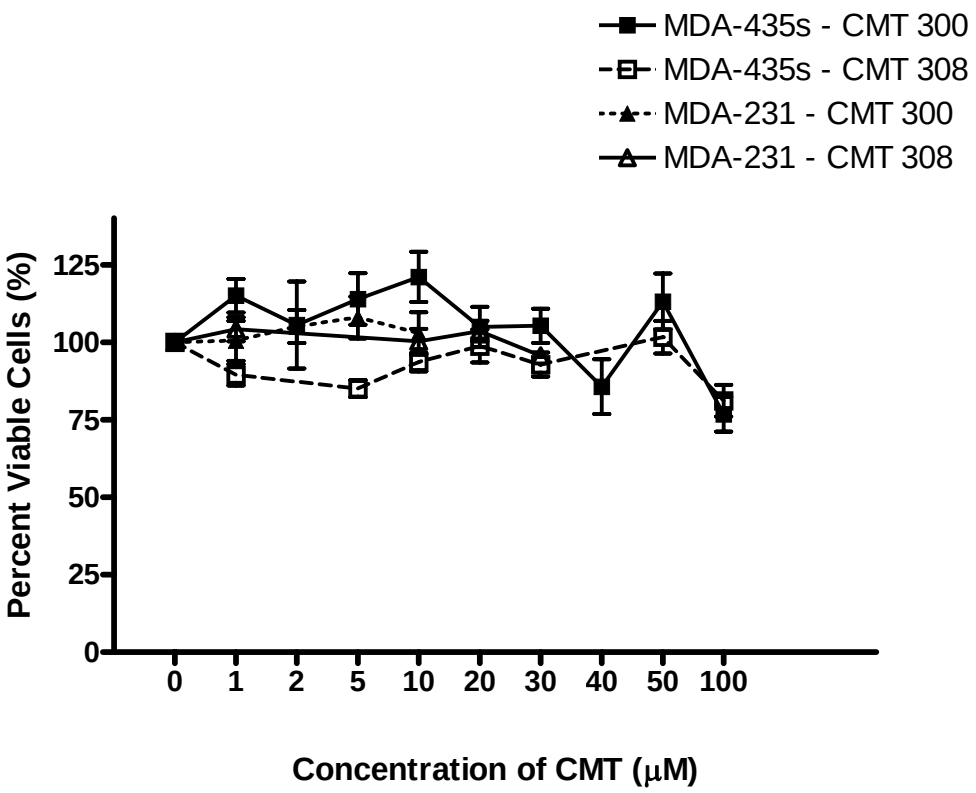
# Figure 15


750
500
250
0
VEGF (pg/ml)
*
**
**
*
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ concentration
Mock
CMT 300 (5 μM)
CMT 308 (20 μM)

# Figure 16


Mock
CMT 300 (5μM)
CMT 300 (20μM)
CMT 308 (20μM)
VEGF (pg/ml)
1400
1200
1000
800
600
400
200
0
***
***
***
***
**
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ Concentration

# Figure 17


MDA-435s - CMT 300
MDA-435s - CMT 308
MDA-231 - CMT 300
MDA-231 - CMT 308
Percent Viable Cells (%)
125
100
75
50
25
0
0
1
2
5
10
20
30
40
50
100
Concentration of CMT (μM)

# Figure 18

**Fig 2B**

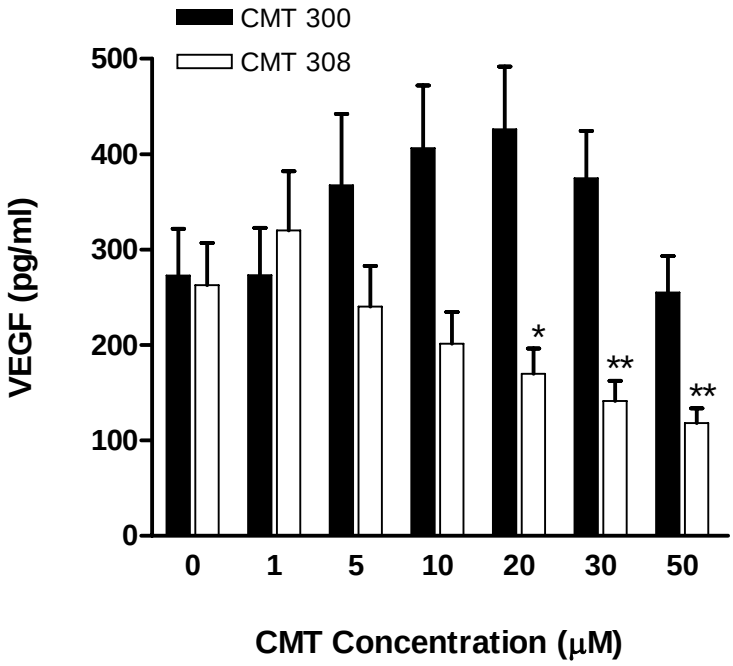
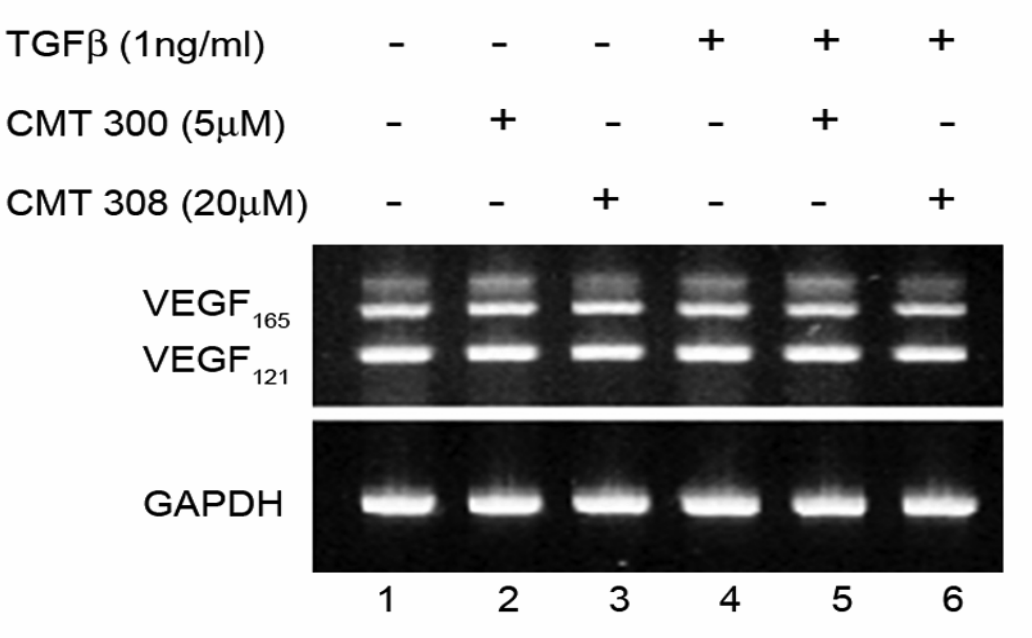
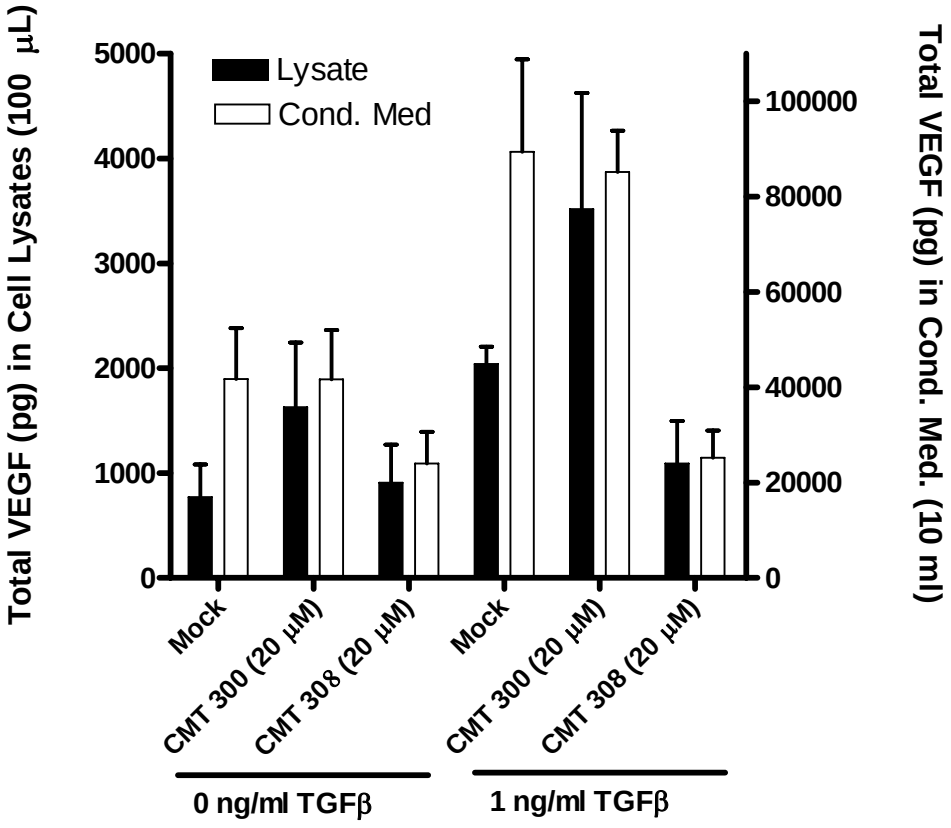


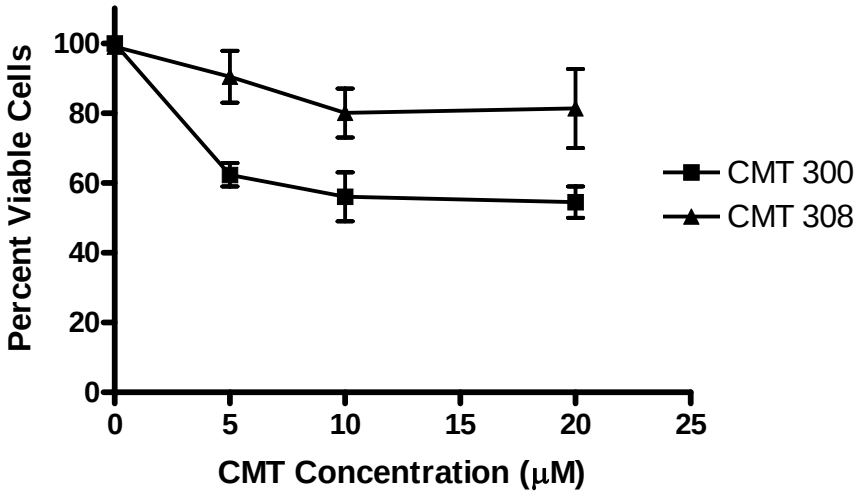
**Figure 19**


TGFβ (1ng/ml) - - - + + +
CMT 300 (5μM) - + - - + -
CMT 308 (20μM) - - + - - +
VEGF165
VEGF121
GAPDH
1 2 3 4 5 6

# Figure 20

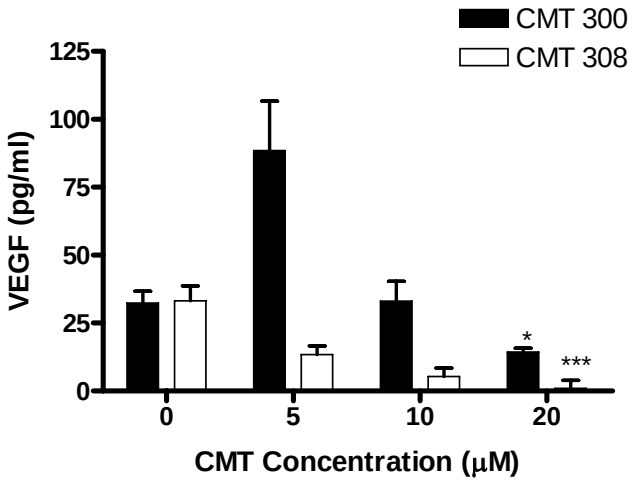

Total VEGF (pg) in Cell Lysates (100 µL)
Total VEGF (pg) in Cond. Med. (10 ml)
Lysate
Cond. Med
5000
4000
3000
2000
1000
0
100000
80000
60000
40000
20000
0
Mock
CMT 300 (20 µM)
CMT 308 (20 µM)
Mock
CMT 300 (20 µM)
CMT 308 (20 µM)
0 ng/ml TGFβ
1 ng/ml TGFβ

# Figure 21

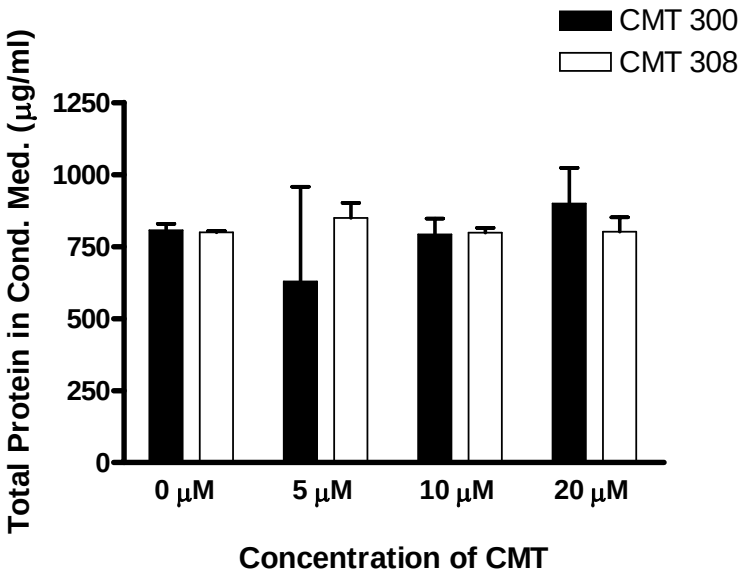

Percent Viable Cells
100
80
60
40
20
0
0
5
10
15
20
25
CMT Concentration (μM)
CMT 300
CMT 308

# Figure 22

**A.**


CMT 300
CMT 308
VEGF (pg/ml)
125
100
75
50
25
0
*
***
0
5
10
20
CMT Concentration (μM)

**B.**


CMT 300
CMT 308
Total Protein in Cond. Med. (μg/ml)
1250
1000
750
500
250
0
0 μM
5 μM
10 μM
20 μM
Concentration of CMT

# Figure 23

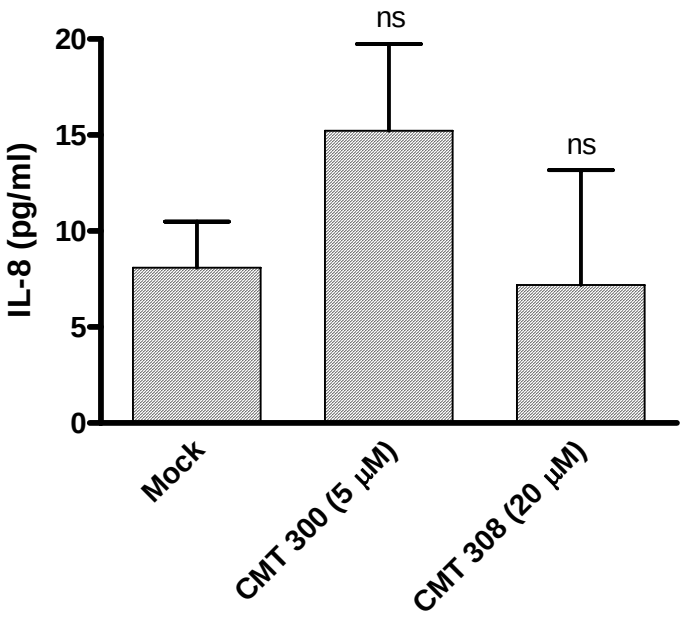
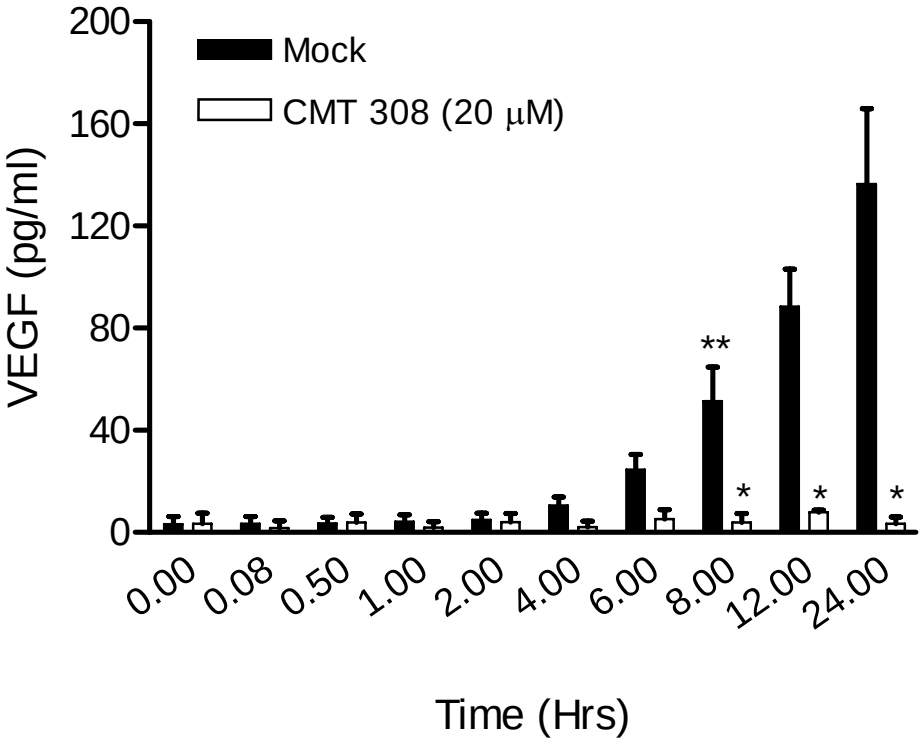


**Figure 24**


200
160
120
80
40
0
VEGF (pg/ml)
Mock
CMT 308 (20 μM)
**
*
*
*
0.00
0.08
0.50
1.00
2.00
4.00
6.00
8.00
12.00
24.00
Time (Hrs)

# Figure 25

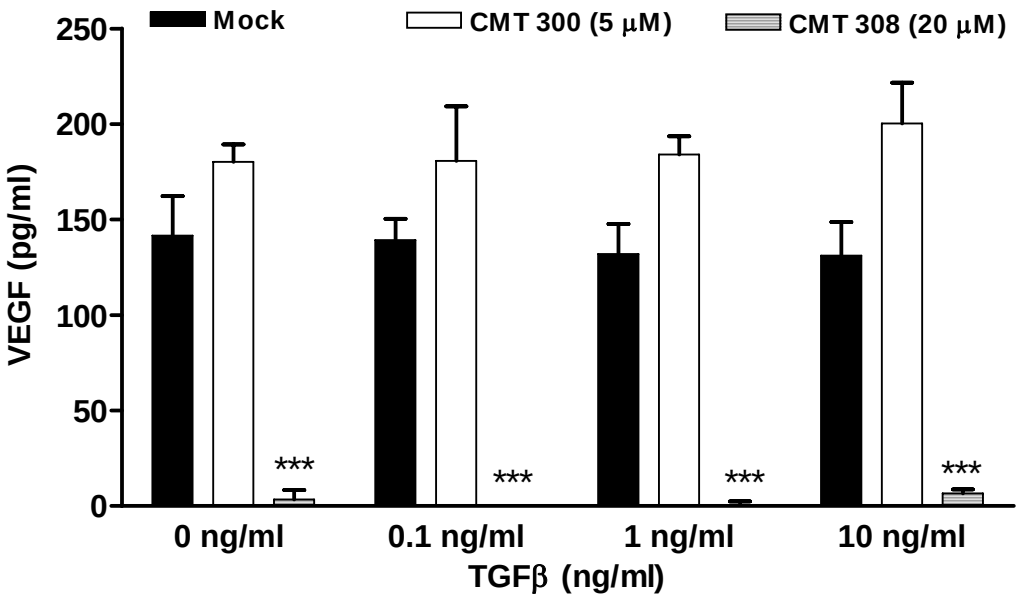
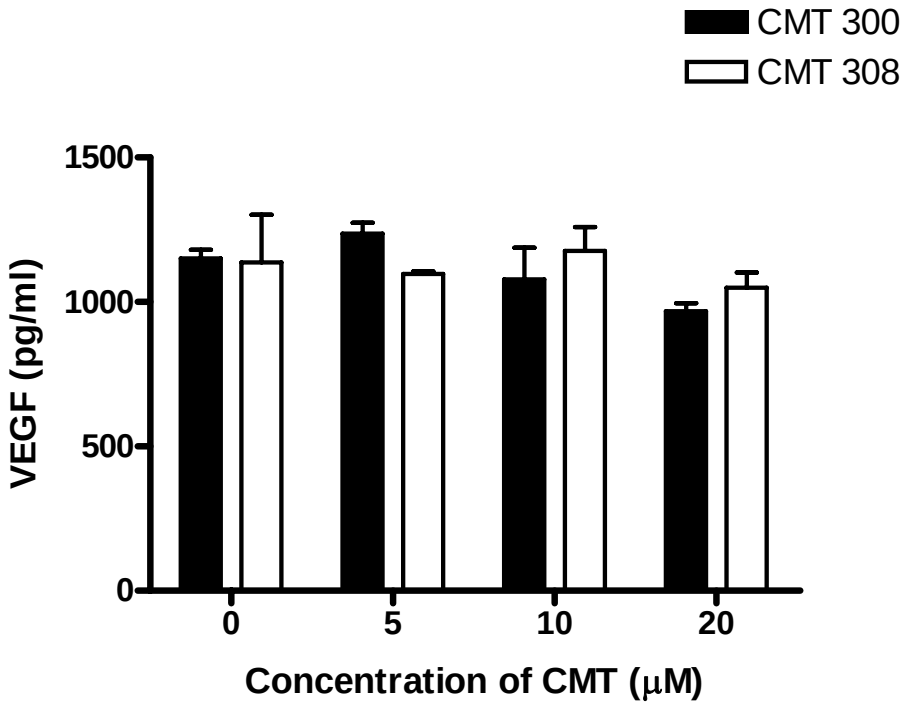
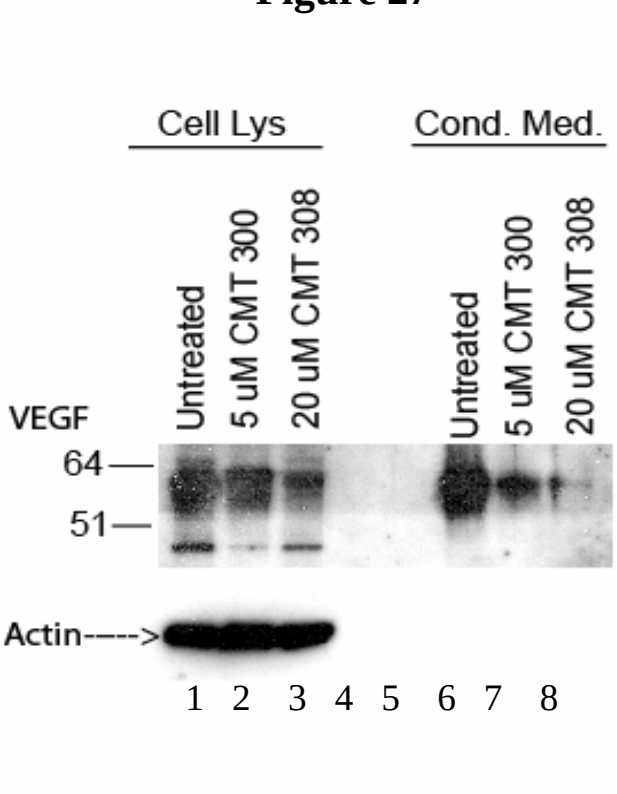

Mock
CMT 300 (5 μM)
CMT 308 (20 μM)
VEGF (pg/ml)
250
200
150
100
50
0
***
***
***
***
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ (ng/ml)

**Figure 26**

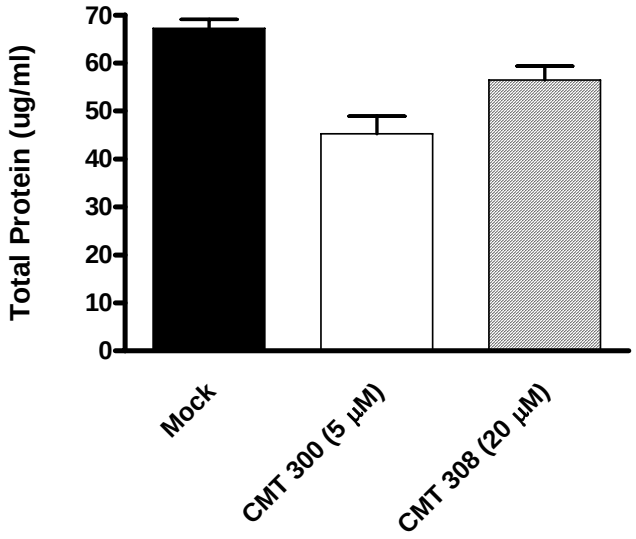

CMT 300
CMT 308
1500
1000
500
0
VEGF (pg/ml)
0
5
10
20
Concentration of CMT (μM)

# Figure 27

**A.**

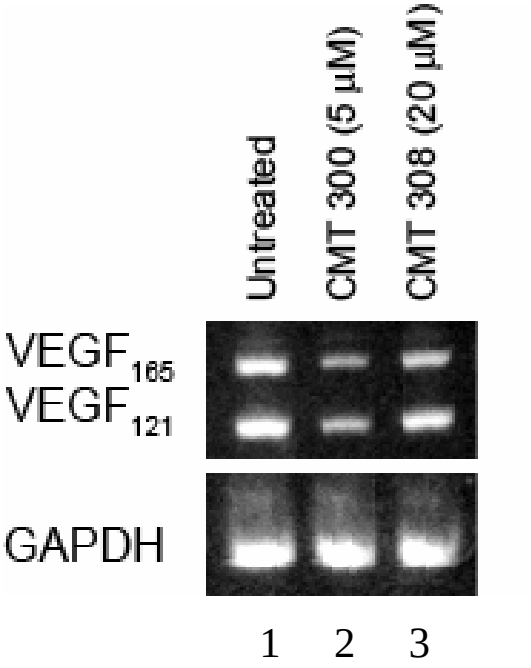

Cell Lys
Cond. Med.
Untreated
5 uM CMT 300
20 uM CMT 308
Untreated
5 uM CMT 300
20 uM CMT 308
VEGF
64
51
Actin---->
1 2 3 4 5 6 7 8

**B.**


Total Protein (ug/ml)
70
60
50
40
30
20
10
0
Mock
CMT 300 (5 μM)
CMT 308 (20 μM)

# Figure 28

**A.**


Untreated
CMT 300 (5 μM)
CMT 308 (20 μM)
VEGF165
VEGF121
GAPDH
1 2 3

**B.**

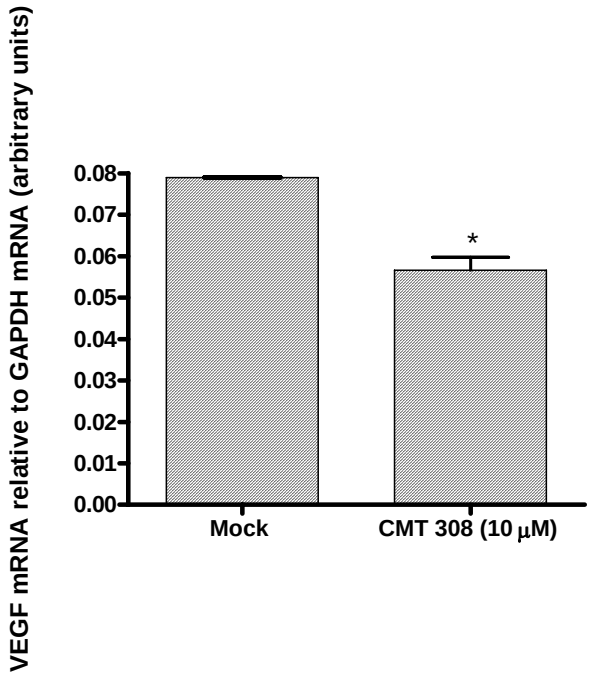
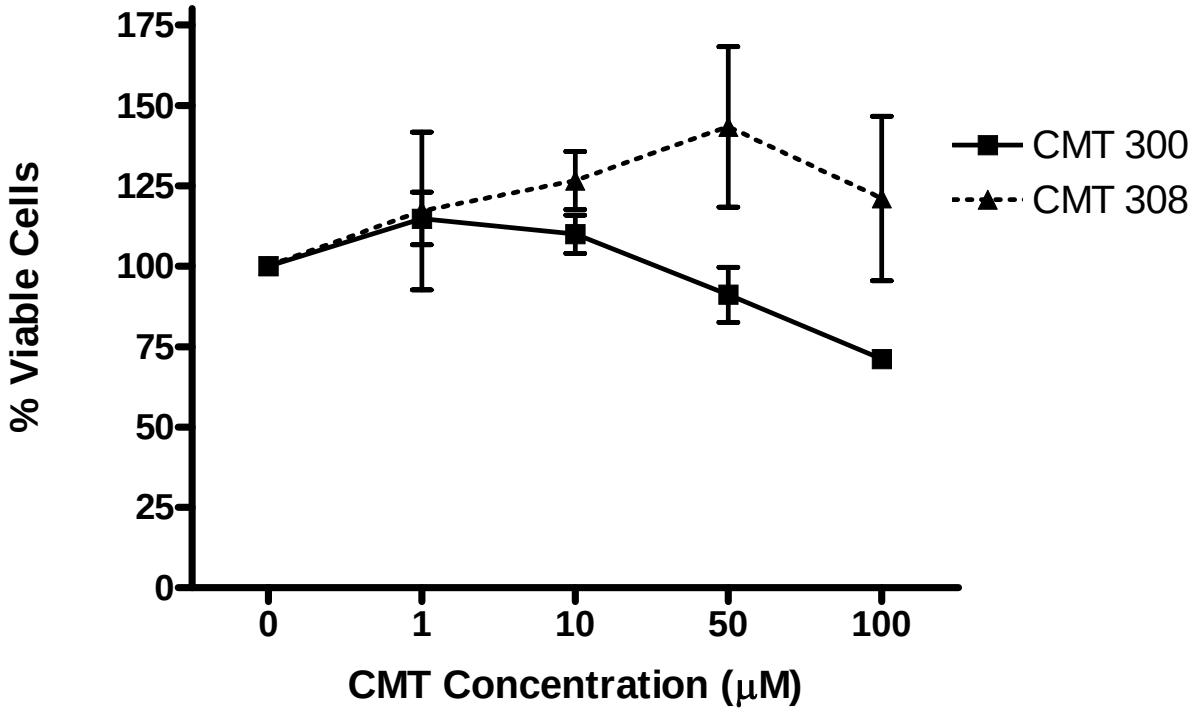
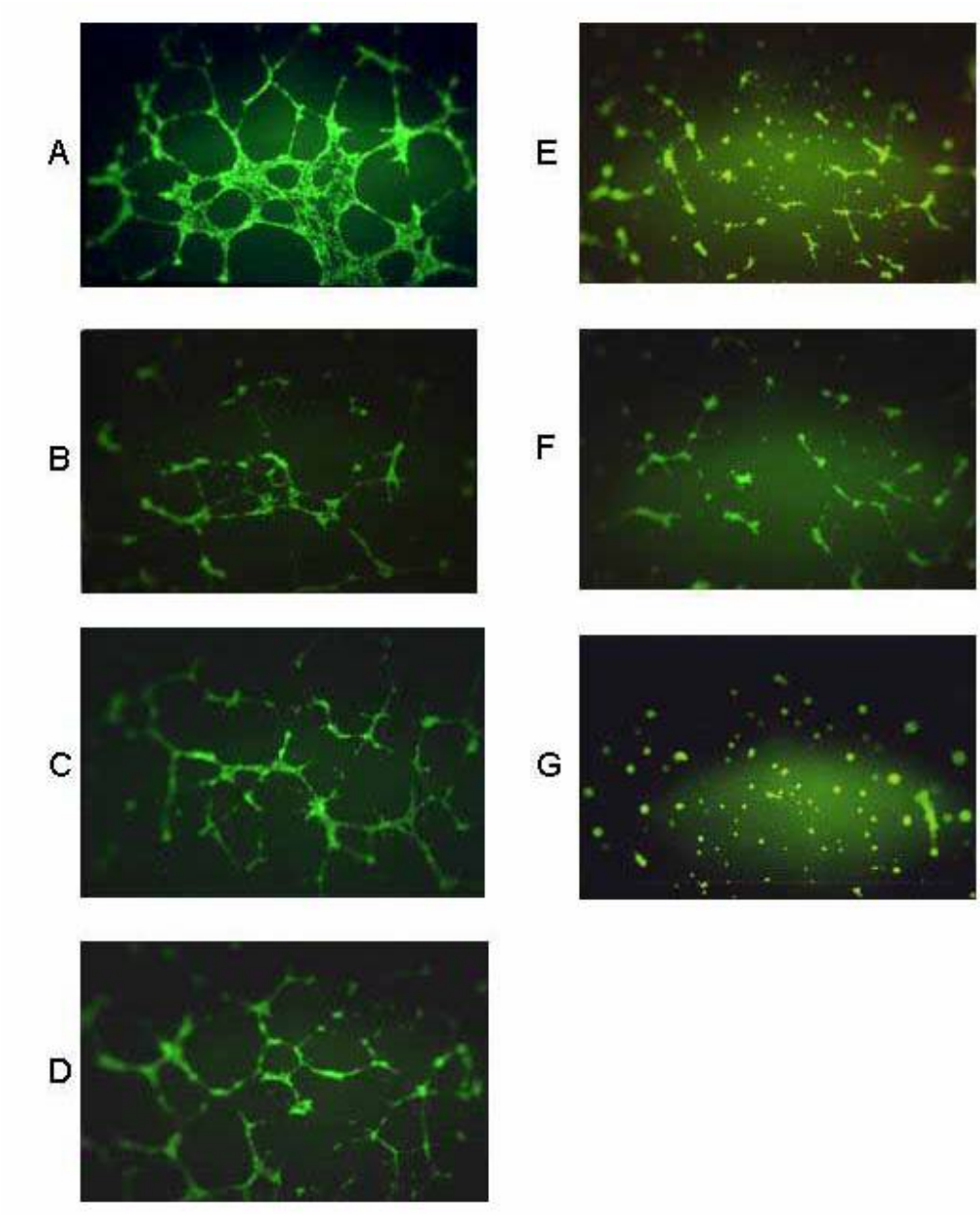

VEGF mRNA relative to GAPDH mRNA (arbitrary units)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
Mock
CMT 308 (10 μM)

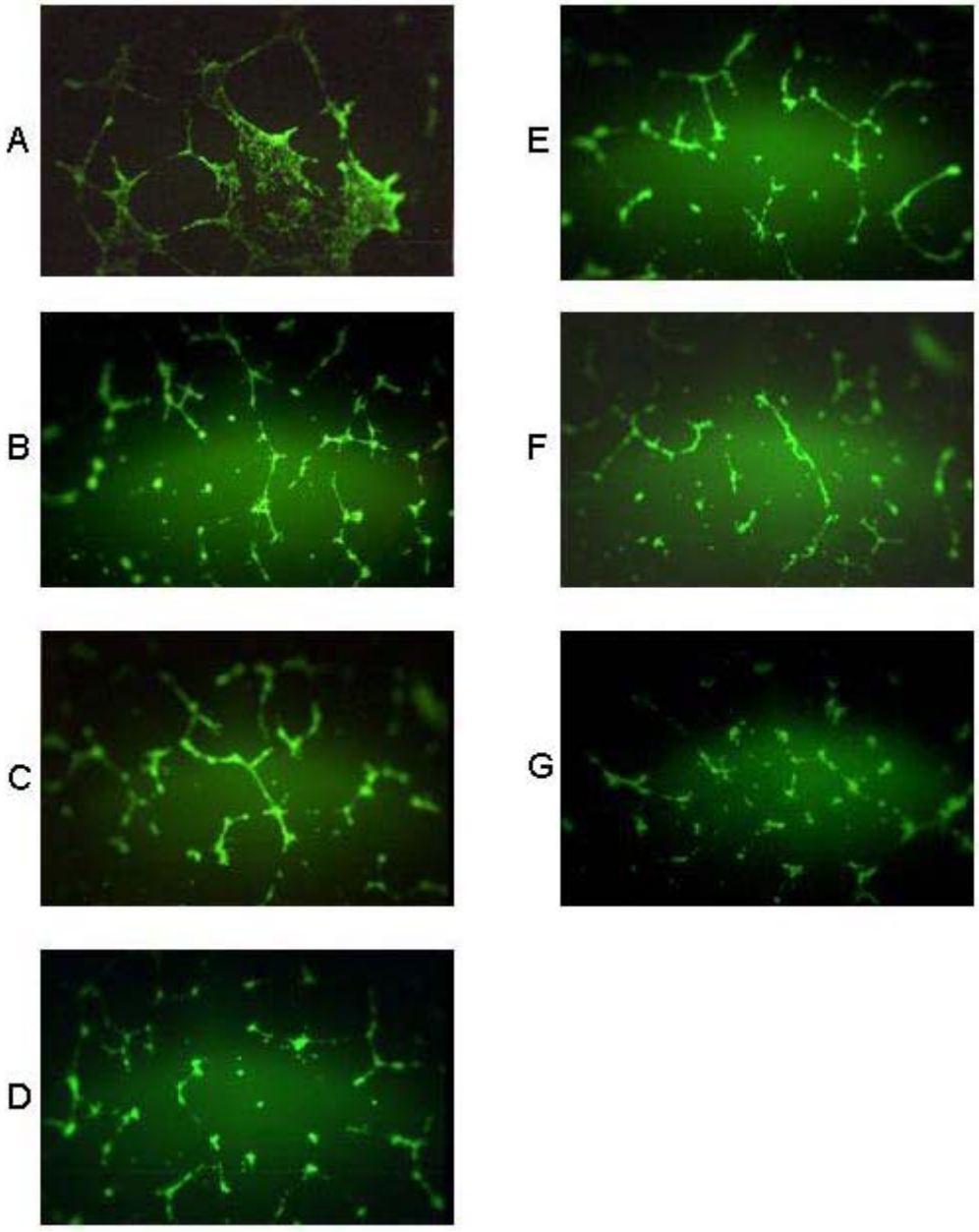
**Figure 29**


175
150
125
100
75
50
25
0
% Viable Cells
0
1
10
50
100
CMT Concentration (μM)
CMT 300
CMT 308

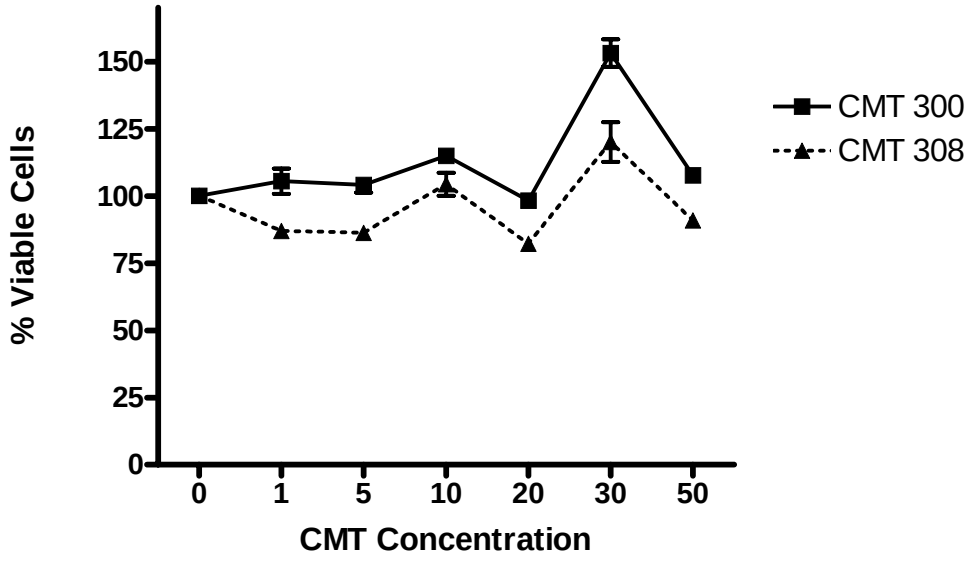
# Figure 30



# Figure 31

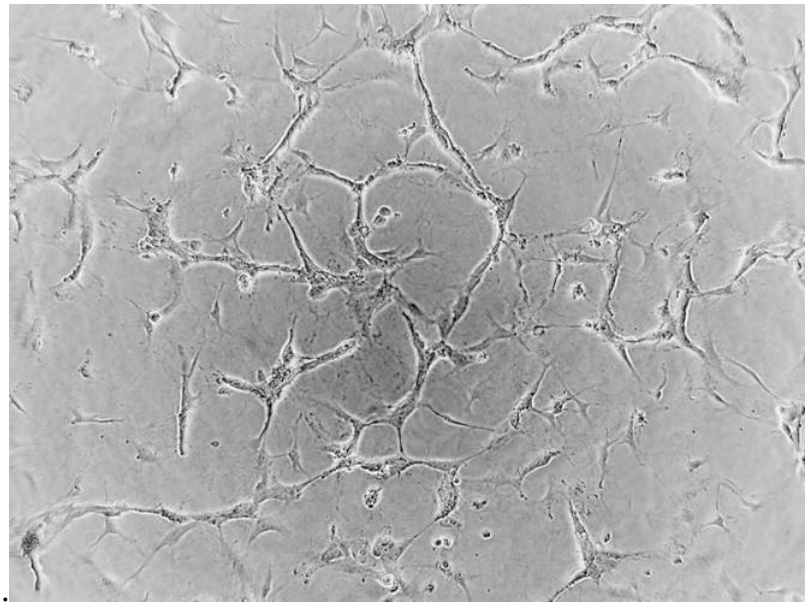


# Figure 32

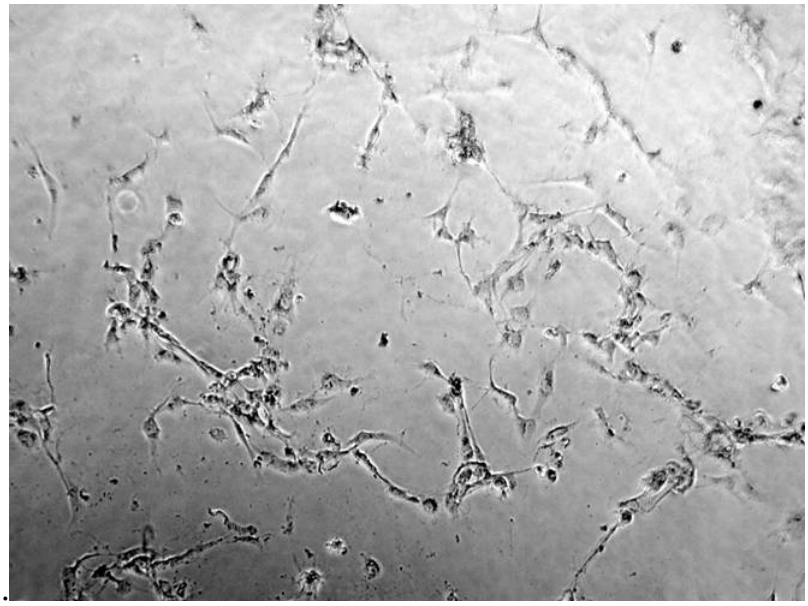

% Viable Cells
150
125
100
75
50
25
0
0
1
5
10
20
30
50
CMT Concentration
CMT 300
CMT 308

# Figure 33

**A.**

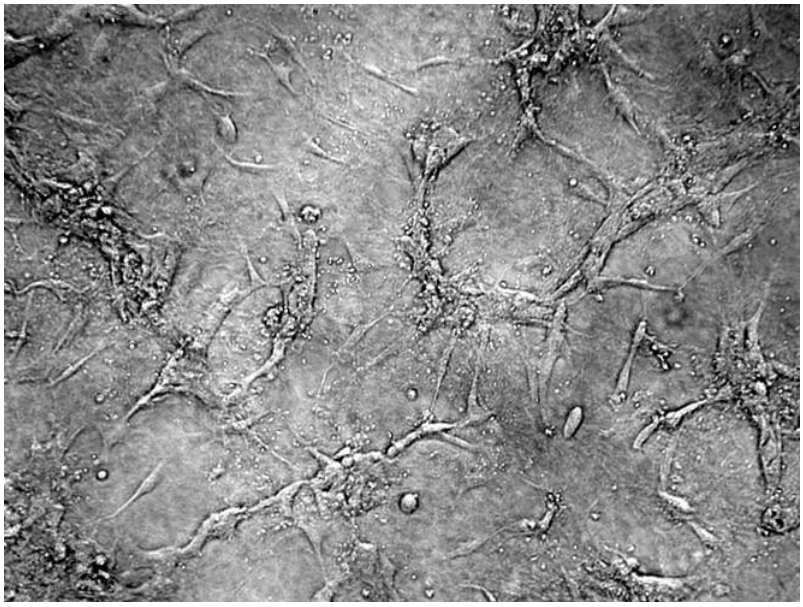


**B.**



# Figure 34

A.



B.

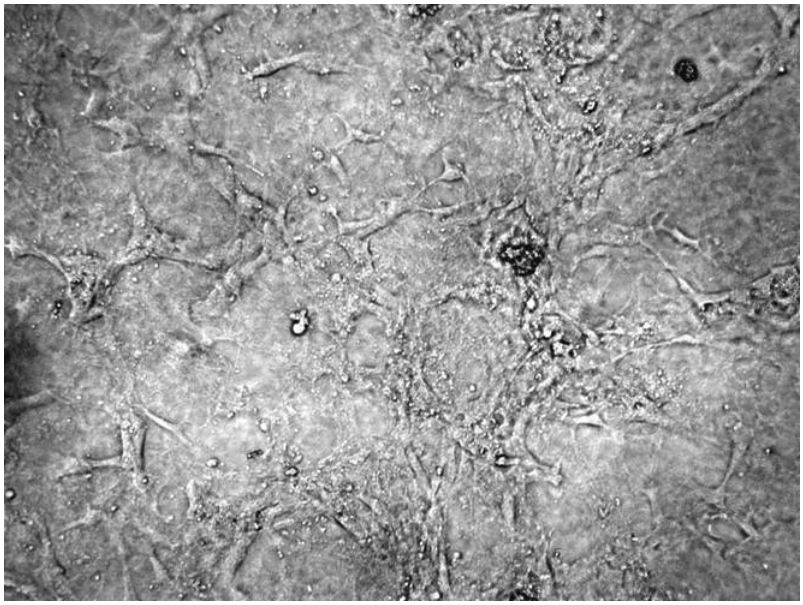
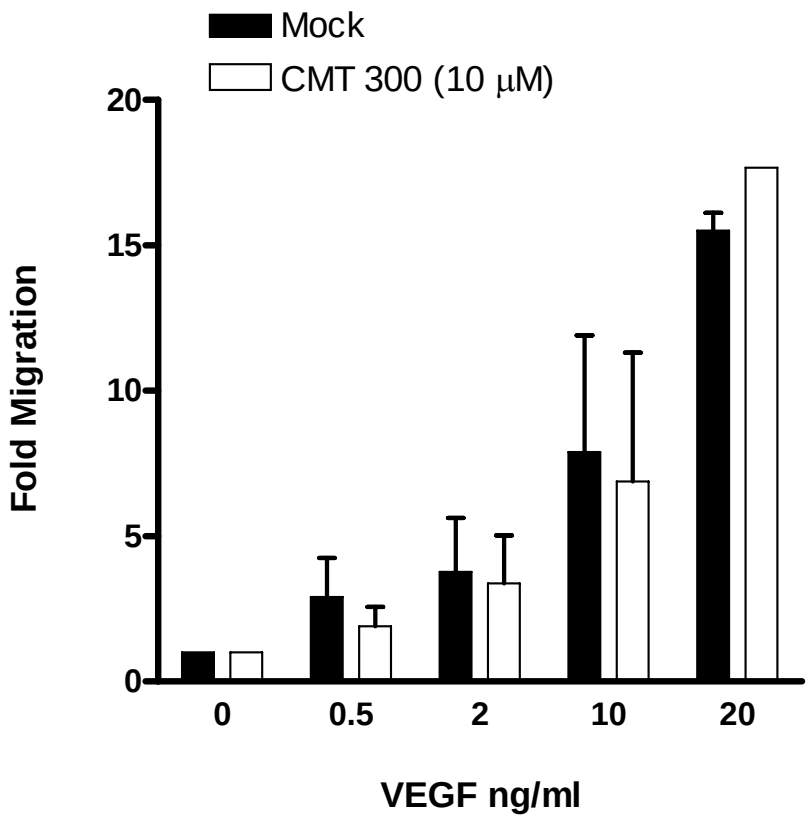
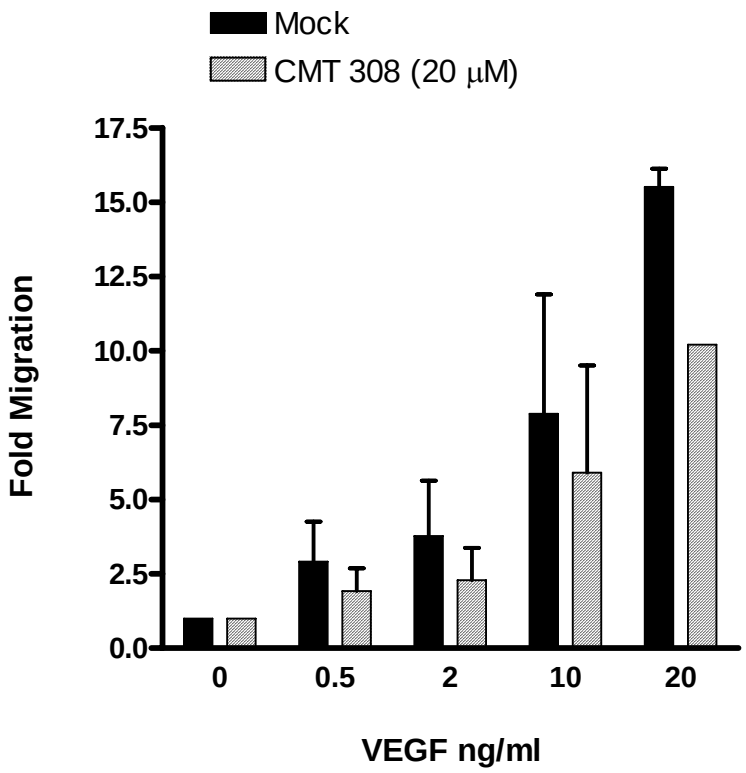


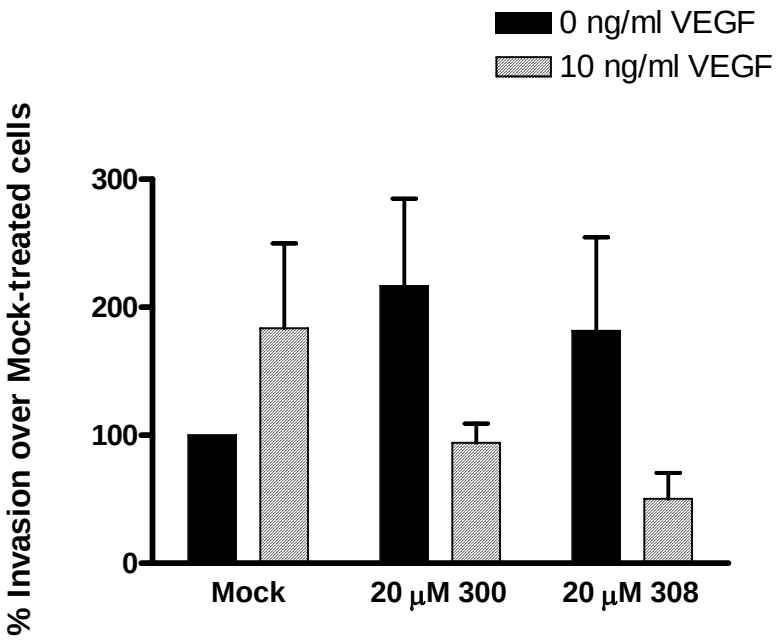
**Figure 35**


Mock
CMT 300 (10 μM)
Fold Migration
20
15
10
5
0
0
0.5
2
10
20
VEGF ng/ml

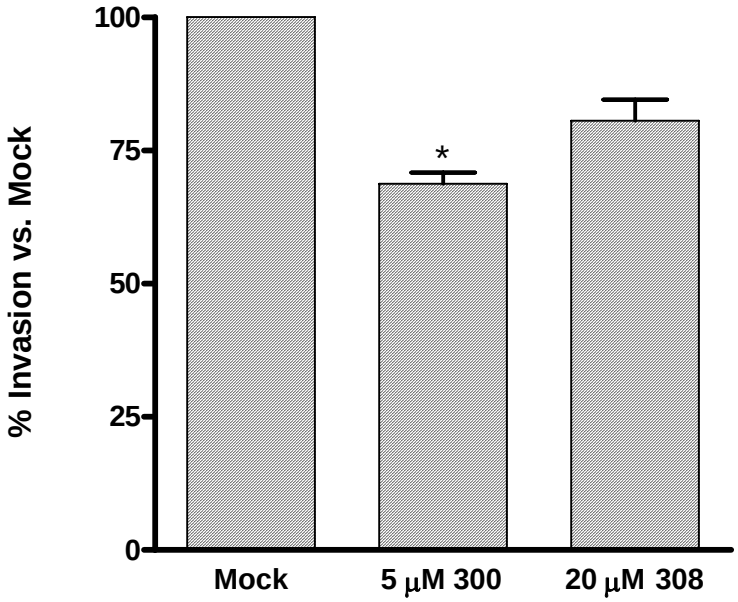
# Figure 36


Mock
CMT 308 (20 μM)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Fold Migration
0
0.5
2
10
20
VEGF ng/ml

# Figure 37


0 ng/ml VEGF
10 ng/ml VEGF
% Invasion over Mock-treated cells
300
200
100
0
Mock
20 μM 300
20 μM 308

# Figure 38



# ANTIANGIOGENIC ACTION OF CHEMICALLY MODIFIED TETRACYCLINES IN BREAST CANCER

**Sanford R. Simon and Mansi Kothari**

Departments of Biochemistry and Pathology, SUNY at Stony Brook, NY 11794-8691

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Control of breast cancer may be achieved by a combination of interventions, including downregulation of the angiogenic response which maintains tumor growth and proliferation. Proangiogenic signals may eminate from the tumor cells themselves as well as from inflammatory cells which infiltrate the tumors. A nonantimicrobial chemically modified tetracycline, 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-300) is currently being evaluated at the National Cancer Institute in Phase I trials on patients with a variety of solid tumors, and has been shown to reduce the angioproliferative response in Kaposi's Sarcoma. The 9-dimethylamino derivative of CMT-300 (CMT-308), induced less photosensitivity in vitro than the parent compound. Accordingly, we have studied the effects of CMT-300 and CMT-308 on two human breast tumor cell lines with different degrees of invasive and metastatic potential as well as a human monocytoid cell line which serves as a model of tumor-infiltrating macrophages. In the first year of research sponsored by this award, we have studied release of Vascular Endothelial Growth Factor (VEGF) by two breast tumor cell lines, MCF-7 (which retains estrogen responsiveness and is not highly invasive) and MDA-MB-231 (which is estrogen insensitive and is highly invasive), using an Enzyme-Linked Immunosorbant Assay (ELISA) for quantitation. Both lines release VEGF at levels which can be augmented in a dose-dependent fashion by Transforming Growth Factor-β (TGF-β). Consistent with the idea that MDA-MB-231 is a model of late stage aggressive breast cancer and MCF-7 is a model of earlier stage cancer, the former line releases higher levels of VEGF than the latter. The levels of VEGF from both lines are diminished when 20 μM CMT-308 (a noncytotoxic dose which is comparable to levels of CMT-300 reached in patients in the NCI Phase I trials) is present during culture. This diminution is especially marked in the presence of TGF-β, suggesting that CMT-308 may be affecting a signal transduction pathway which is activated in these tumor cell lines by TGF-β. Levels of VEGF released by both breast tumor lines are diminished much more by CMT-308 than by CMT-300. Because highly vascularized tumors are often also infiltrated with inflammatory cells, we examined the effects of the CMTs on VEGF production by a highly differentiated monocytoid cell line, Mono Mac 6. This cell line releases high levels of VEGF which are not affected by TGF-β, but 20 μM CMT-308 effectively abrogates all VEGF release. The results on VEGF release indicate that the CMTs may have utility in management of breast cancer by diminishing the pro-angiogenic signals released by the tumors and by infiltrating mononuclear cells. Because the CMTs have been found to be safe and well tolerated in cancer patients as well as normal volunteers, they have promise for rapid development as components of comprehensive therapeutic strategies for breast cancer management.

Supported by the U.S. Army- Medical Research and Materiel Command under DAMD17-01-1-0346.

# ANTIANGIOGENIC ACTION OF CHEMICALLY MODIFIED TETRACYCLINES IN BREAST CANCER

**Sanford R. Simon And Mansi Kothari**
Departments of Biochemistry and Pathology, SUNY at Stony Brook, NY 11794-8691
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The long term objective of this project is to evaluate two nonantimicrobial chemically modified tetracyclines (CMTs), CMT-300 and CMT-308, as inhibitors of the angiogenic response which maintains breast tumor growth and proliferation. CMT-300 continues to show efficacy in NCI-sponsored Phase I/II trials for reduction of the angioproliferative lesions in Kaposi's sarcoma. In the first two years of research sponsored by this award, we studied effects of these two CMTs on release of Vascular Endothelial Growth Factor (VEGF) by two breast tumor cell lines, MCF-7 (which retains estrogen responsiveness and is not highly invasive) and MDA-MB-231 (estrogen insensitive and highly invasive), using an Enzyme-Linked Immunosorbant Assay (ELISA) for quantitation. In the two years since we first presented our results at the Era of Hope Conference, we have expanded our studies on release of VEGF from breast tumor cell lines to include two additional cell lines, MDA-MB-453 and MDA-MB-435s, which are more invasive than MCF-7. CMT-300 stimulated release of VEGF from the cell lines except at the high dose of 50 μM, but CMT-308 inhibited release in a dose dependent fashion. At 20 μM CMT-308, a dose comparable to the CMT-300 levels in the plasma of Kaposi's sarcoma patients, the release of VEGF from MCF-7 cells was suppressed virtually completely for up to 8 hours. TGF-β augmented VEGF release by the tumor cell lines, but this enhanced release was also inhibited by CMT-308 in a dose-dependent fashion. Neither CMT triggered cytotoxicity in any breast tumor cell line at doses up to 50 μM. The diminished VEGF levels could be ascribed to inhibition of synthesis and/or secretion, rather than degradation of pre-existing growth factor. Steady-state levels of the mRNAs for multiple VEGF species, measured by PCR, were unaffected by TGF-β or CMTs, whereas intracellular VEGF protein pools were diminished by the CMTs, consistent with action at the posttranscriptional level. Release of VEGF from the human monocytoid line Mono Mac 6, a model of tumor-infiltrating macrophages, was inhibited by >50% by 5 μM CMT-308 and by >90% by 20 μM CMT-308: Intracellular VEGF pools in Mono Mac 6 cells were also diminished by CMT-308, although by less than secretion; there was no significant effect of CMTs on the multiple VEGF mRNA species. In the absence of cytotoxicity, both CMT-300 and CMT-308 inhibited tube formation by human microvascular endothelial cells in a thick coating of Matrigel with IC50 values in the low μM range. Both CMTs also visibly diminished the morphogenic transition triggered by addition of type I collagen to endothelial cells, although by less than when cells were plated on Matrigel. Migration of endothelial cells through fibronectin-coated porous cell culture inserts was also inhibited by both CMTs. These results offer further support for the potential use of CMTs as antiangiogenic agents in management of breast cancer.

*Original work supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0346 and current work supported by .*

# Chemically Modified Tetracyclines Inhibit VEGF Secretion by Breast Cancer Cell Lines:

**Mansi Kothari and Sanford R. Simon**

Department of Pathology

State University of New York at Stony Brook

Stony Brook, NY 11794-8691

## Abstract

Chemically Modified Tetracyclines (CMTs) are antiproteolytic agents that have been shown to inhibit tumor invasiveness and metastasis. CMT 300 has shown promise in phase I clinical trials in patients with Kaposi's Sarcoma, which is characterized by over-production of the pro-angiogenic cytokine vascular endothelial growth factor (VEGF). In this study, we report a novel activity of CMT 308, a 9-amino derivative of CMT 300, on reducing levels of VEGF secreted by breast cancer cell lines. CMT 308, at sub-cytotoxic concentrations, reduced basal levels of secreted VEGF in the poorly invasive MCF-7 cell line as well as the more aggressively invasive MDA-MB-435s cell line in a dose-dependent manner. In addition, CMT 308 also reduced Transforming Growth Factor β (TGFβ)-induced VEGF secretion in both cell lines. While VEGF could be detected in the conditioned media of untreated MCF-7 cells within 4 hours, when MCF-7 cells were treated with CMT 308, no VEGF was secreted at the earliest detectable time point, and levels of secreted VEGF in CMT 308-treated cells remained undetectable up to 8 hours. CMT 308 diminished secretion of VEGF from MCF-7 cells up to 8 hours regardless of previous time in culture. CMT 308 did not reduce the levels of basal VEGF mRNA in either cell line. However, CMT 308 did reduce pools of total intracellular VEGF protein. Although TGFβ stimulated an increase in VEGF levels in the conditioned media as well as in the cytoplasm, TGFβ treatment did not increase VEGF mRNA levels. Thus, augmented expression of VEGF protein by breast cancer cell lines in the presence of TGFβ appears to involve upregulation at a step beyond transcription. Moreover, the data strongly indicate that in these breast cancer cell lines, CMT 308 reduces VEGF secretion by targeting some post-transcriptional event. The capacity of CMT 308 to diminish levels of a major pro-angiogenic signal makes the nonantimicrobial tetracycline derivative an attractive candidate for anti-angiogenic therapy in management of breast cancer.

# Introduction

Breast cancer is the most frequently diagnosed non-skin cancer and ranks second among cancer deaths in women after lung cancer. Sustenance, progression and metastasis of breast and other tumors require elaboration of new micro-vessels from pre-existing vasculature through a process known as angiogenesis (1, 2). Angiogenesis is a consequence of a delicate balance between pro- and anti-angiogenic drives. Several pro-angiogenic molecules have been identified, including Vascular Endothelial Growth Factor (VEGF), basic fibroblast growth factor (bFGF), interleukin 8 (IL-8) and Transforming Growth Factor β (TGFβ) (3-5). The most potent of these is VEGF, which is specifically mitogenic for endothelial cells that line blood vessel walls (6). While molecules such as TGFβ are considered indirect inducers of angiogenesis, their role in promoting angiogenesis makes them an important target for anti-angiogenesis therapy (7, 8). One of the tumor-promoting functions of TGFβ is to induce angiogenesis by increasing VEGF production (9, 10) in tumor cells. Moreover, there is evidence of the involvement of TGFβ in breast cancer. TGFβ levels are increased in the plasma of breast cancer patients, and these high TGFβ levels correlate with high VEGF levels in the patients tested (11). Hence, strategies that inhibit VEGF production through regulatory molecules such as TGFβ have become viable candidates for anti-angiogenic therapy.

The focus of this study is a group of chemically modified tetracyclines (CMTs), non-antimicrobial tetracycline analogs that have anti-proteolytic properties (12). The anti-proteolytic activity of CMTs may be responsible for their capacity to inhibit the invasiveness of many tumor cells including cells of glioblastoma, breast (13), prostate (14-16), colon (17, 18) and melanoma cells (19). Additionally, the capacity of CMTs to induce apoptosis in a variety of cell lines has been proposed as a basis of their antitumor activity. Cells undergoing apoptosis in the presence of CMTs include macrophage tumor cell lines (20), leukemia cell lines (21) and prostate tumor cell lines (15). Previous studies on CMTs have alluded to the antiangiogenic capacity of this

subset of the tetracycline family of molecules. CMT 300 (6-deoxy-6-demethyl-4-dedimethylaminotetracycline), a modified tetracycline inhibits the formation of small tubules by human umbilical vein endothelial cells (HUVEC) (22). CMT-1, which lacks the C-4 dimethylamino group on tetracycline appears to be more effective than minocycline in inhibiting endothelial cell proliferation (23). Additionally, both, CMT 1 and CMT 8 (6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline) inhibit the secretion of PMA-induced matrix metalloprotease 9 (MMP 9) expression in HUVEC (24). Secretion of matrix degrading enzymes such as MMP-9 by HUVEC is thought to be important for formation of new microvessels during angiogenesis. Further evidence for the antiangiogenic effect of CMTs comes from the results of a phase I clinical trial with CMT 300 on patients with Kaposi's Sarcoma (KS). KS is generally considered a disease of overproduction of VEGF due to the presence of latent Human Herpes Virus 8 (HHV8) genes (25). When treated with CMT 300, patients with KS had an overall response rate of 44 percent (26) and their lesions had visibly regressed (27). Current anti-angiogenic therapies such as the recently approved drug Avastin involve neutralizing antibodies against VEGF protein. In this study, we report a novel capacity of CMT 308, a non-phoototoxic, 9-amino derivative of CMT 300, to reduce secreted levels of the pro-angiogenic cytokine, VEGF, through a post-transcriptional modulatory mechanism, leading to diminished release of VEGF by breast cancer cells.

## Materials & Methods

Reagents:

All reagents unless otherwise specified were purchased from Sigma (St. Louis, MO). MCF-7 cells, MDA-MB-435s cells, and Eagle's Minimum Essential Medium (MEM) were purchased from ATCC (Manassas, VA). Dulbecco's Minimum Essential Medium (DMEM) - low glucose and Fetal Bovine Serum (FBS) were purchased from Hyclone (Logan, UT). TGFβ1 isolated from human platelets was purchased from Calbiochem (LaJolla, CA).

Cell Culture: MCF-7 cells were maintained in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) supplemented with 0.01 mg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich). MDA-MB-435s cells were maintained in DMEM-low glucose supplemented with 10% FBS and 1% penicillin/streptomycin.

Cytotoxicity assays: Fresh serum-free medium was added to cells followed by addition of MTS solution (Promega, Madison, WI). Cells were then incubated at $37^0$C for 1-4h, and absorbance was recorded at 490nm.

VEGF Quantitation in Conditioned Medium: MCF-7 & MDA-MB-435s cells (ATCC) were plated at $10^5$ cells/well in 1ml complete medium in 48-well plates and incubated overnight. The next day, medium was aspirated and replaced with fresh serum-free medium. Where indicated, TGFβ1 diluted in serum-free medium was added at various indicated concentrations. CMTs were diluted in DMSO and added to wells so that the final concentration of DMSO was 0.5%. Cells were further incubated for 24h. Conditioned medium was then collected and stored at $-20^0$C. The VEGF Quantikine ELISA assay (R&D Systems, Minneapolis, MN) was used to assay VEGF in the conditioned medium from each sample. Loss of viability of the cells after the various treatments was evaluated by addition of MTS to a set of identically treated wells in the same plate.

VEGF Quantitation in Cell Lysates:

$10^6$ MDA-MB-435s cells plated in 10 ml serum-free medium were treated for 24 hours with agents being tested or with DMSO as a vehicle control. Conditioned medium from the cells was collected for VEGF analysis. Cells were then trypsinized and lysed using 100 µL lysis buffer (150mM sodium chloride, 40mM Tris, 10% Glycerol, 0.1% NP40, 5mM sodium fluoride, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, pH 7.8) with Complete Mini Protease Inhibitor Cocktail (Roche Biochemicals, Indianapolis, IN). Lysates were subjected to three freeze-thaw cycles centrifuged at 10,000 rpm for 10 minutes, and the supernatants collected for VEGF analysis. The VEGF Quantikine ELISA assay (R&D Systems, Minneapolis, MN) was used to assay VEGF in the lysates and conditioned medium from each sample. 20 uL aliquots of cell lysate or 150 uL aliquots of conditioned medium were routinely used for assay. Total protein levels were simultaneously evaluated in cell lysates and conditioned media using the BCA protein assay (Pierce, Rockford, IL). Total VEGF levels in cell lysates and conditioned media were calculated after correcting for differences in dilution.

RT-PCR for VEGF mRNA : TRI reagent (Molecular Research Center Inc., Cincinnati, OH) was used as per manufacturer's instructions to isolate total RNA. RT-PCR was performed using 1-step RT-PCR kit (Qiagen, Valencia, CA). Equal quantities of total RNA were assayed using a PCR machine. Primers for VEGF used were chosen such that they recognize and distinguish between VEGF 189 (479 bp), VEGF 165 (407 bp), VEGF 145 (347 bp),VEGF 121 (275 bp) (28). Forward: 5'-CTGGTGGACATCTTCCAGGAGTA-3' and Reverse: 5'-CTCACCGCCTCGGCTTGTCACA-3'. The human GAPDH control primer set (Clontech, Palo Alto, CA) was used as a control for the PCR.

Quantitative Assay for VEGF mRNA: RNA samples collected above were also quantitated using the Quantikine mRNA assay (R & D Systems, Minneapolis, MN) according to manufacturer's instructions. Briefly, the assay is an ELISA-based, colorimetric microplate assay, where 4 µg of total RNA from each sample was

hybridized with either VEGF-specific or GAPDH-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in a microplate. The hybridization solution was then transferred to a streptavidin-coated microplate and the RNA/probe hybrid captured. An antidigoxigenin alkaline phosphatase conjugate was added for detection, followed by a substrate solution. An amplifier solution was then added and color developed in proportion to the amount of gene-specific mRNA (R & D Systems Minneapolis, MN) in the total RNA sample. Color development was stopped and the intensity of the color was measured spectrophotometrically.

Data Analysis and Statistics: A minimum of three experiments were performed for each assay. Results were analyzed for group means using ANOVA followed by Newman-Keul's multiple comparison post-test employing Instat™ statistical analysis software (GraphPad, San Diego, CA).

## Results

### CMT 308 reduces basal as well as TGFβ-induced levels of VEGF from poorly invasive and aggressively invasive breast cancer cell lines

The angiogenic potential of tumor cells may vary depending on the invasiveness and stage of the cancer. In order to determine the responsiveness of tumor cells at either end of the spectrum of metastatic potential, we evaluated two different breast cancer cell lines that represent early and late stages of breast cancer. MCF-7 is a cell line that is estrogen responsive and poorly invasive and is considered a model for early stages of breast cancer, while the MDA-MB-435s cell line is estrogen unresponsive, more invasive and is considered a model for later stages of breast cancer. Equivalent numbers of MCF-7 cells secrete lower basal levels of VEGF than MDA-MB-435s cells, which is reflective of their relative invasive and metastatic potential (Fig 1A, B). In addition to this basal VEGF secretion, secretion of VEGF from tumor cells can be augmented in the presence of several growth factors. TGFβ is an indirect angiogenic growth factor that has been reported to induce VEGF secretion by several different cell types (7, 9, 10). In order to evaluate whether TGFβ could also stimulate VEGF secretion in breast cancer cell lines, MCF-7 and MDA-MB-435s cells were treated with varying doses of TGFβ. In MCF-7 cells, TGFβ at 0.1, 1 and 10 ng/ml increased VEGF secretion by 20%, 42% and 57% respectively over the levels released by untreated cells (Fig 1A). Not only did the more invasive MDA-MB-435s cell line produce higher basal levels of VEGF than MCF-7 cells, but this line also had a significantly greater response to TGFβ. At TGFβ doses of 0.1, 1 and 10 ng/ml, levels of VEGF secreted by MDA-MB-435s cells were increased by 75%, 376% and 292% respectively (Fig 1B).

Inhibition of secretion of VEGF by two CMTs, CMT-300 and CMT-308, was evaluated in both cell lines. CMT 300 at 20 μM did not inhibit basal VEGF secretion by MCF-7 cells; instead, it stimulated basal VEGF secretion by 130% over basal levels (Fig 1A). In contrast to the stimulation of VEGF production by CMT 300, in the presence of 20 μM CMT-308, basal levels of VEGF released by MCF-7 cells were diminished by as

much as 50%. Addition of 20 μM CMT 308 also resulted in a 50% diminution of VEGF secretion in MCF-7 cells treated with 0.1 and 1 ng/ml TGFβ, and a 42% reduction in cells treated with 10 ng/ml TGFβ. TGFβ-stimulated VEGF secretion from MCF-7 cells was not diminished by concentrations of CMT 300 comparable to the doses of CMT 308 that had produced significant inhibition of VEGF production.

A more pronounced effect was observed in MDA-MB-435s cells. Similar to our observations with MCF-7 cells, addition of 20 μM CMT 300 could not appreciably reduce VEGF secretion by MDA-MB-435s cells in the absence of TGFβ (Fig 1B). In MDA-MB-435s cells treated with TGFβ, CMT 300 had only minimal effect on secretion of VEGF. However, addition of 20 μM CMT 308 resulted in a 60% diminution in the basal levels of VEGF released by the more invasive MDA-MB-435s cell line in the absence of TGFβ. Furthermore, addition of 20 μM CMT 308 to cells stimulated with 0.1 and 1 ng/ml TGFβ resulted in 60% and 75% diminution of VEGF secretion respectively, while at 10 ng/ml TGFβ, CMT 308 caused a 40% reduction in VEGF secretion (Fig 1B).

Treatment of MCF-7 cells with IGF-I or IGF-II, which have been reported to contribute to pro-angiogenic activity in other systems, did not result in increased VEGF secretion. Additionally, CMT 308 reduced VEGF in IGF-I treated cells to the same extent as it did basal secretion (data not shown). These experiments indicate that CMT 308 was more effective than CMT 300 in reducing levels of secreted VEGF in both the poorly invasive and the highly invasive breast cancer cell lines. Moreover, at least one step in modulation of VEGF secretion specifically induced by TGFβ is also sensitive to inhibition by CMT 308.

**Dose dependent effects of CMT 308 on VEGF secreted by breast tumor cell lines**

In order to understand the mechanism by which culture of breast tumor lines in the presence of CMT-308 resulted in diminished release of VEGF, we first determined the dose dependence of the CMT 308-mediated diminution. MCF-7 cells were treated with 1 ng/ml TGFβ and varying doses of CMT 308. CMT 308 at 20 μM reduced VEGF

secretion by 42% while at 50 μM it reduced VEGF secretion by 82% (Fig 2A). As noted above in Fig 1A, treatment with 20 μM CMT-300 caused a stimulation of basal VEGF secretion in the MCF-7 cell line. Thus, in MCF-7 cells, CMT 308 inhibited TGFβ-induced VEGF secretion in a dose-dependent manner, while CMT 300 did not. The differing effects of the two CMTs were also observed with the more invasive MDA-MB-435s cells. Doses of CMT 300 up to 30 μM had a stimulatory effect on basal VEGF secretion, while in the presence of 50 μM CMT 300 VEGF secretion fell back to basal levels. In contrast, CMT 308 caused dose-dependent decreases in VEGF secretion from MDA-MB-435s cells in the absence of TGFβ, except at the lowest dose (1 μM) of CMT-308 tested (Fig 2B). At 5 μM CMT-308, basal VEGF levels were diminished by 9%; this trend continued up to 50 μM at which point VEGF levels were diminished by 55%. Thus, CMT 308 diminished not only basal levels of VEGF secretion from breast tumor lines in a dose dependent manner as seen in the MDA-MB-435s cells, but also TGFβ-induced VEGF secretion as seen in MCF-7 cells.

**Cytotoxic effects of CMTs on breast cancer cell lines**

It has been known for some time that tetracyclines impair mitochondrial protein synthesis (29). Inhibition of mitochondrial protein synthesis over several cell cycles results in the arrest of cellular proliferation. Tetracyclines are therefore considered cytostatic for some cell types (29, 30). To determine whether the reduction in VEGF secretion by CMT 308 was a result of its cytotoxicity to breast cancer cell lines, we treated MCF-7 and MDA-MB-435s cells with varying concentrations of either CMT 300 or CMT 308. Cytotoxicity was examined using the MTS assay, which evaluates levels of mitochondrial dehydrogenase activity associated only with viable cells. CMT 300 up to concentrations of 30 μM produced no cytotoxicity on MCF-7 cells (Fig 3). However, at lower concentrations from 5 μM to 20 μM CMT 300 appeared to stimulate mitochondrial dehydrogenase activity by up to 50%, as judged by increased conversion of the tetrazolium salt to its formazan. In the MDA-MB-435s cells as well, CMT 300 from 5 μM to 20 μM had an apparent stimulatory effect on mitochondrial dehydrogenase

activity (Fig 3). Thus at low concentrations, CMT 300 appeared to induce metabolic activation of the cells.

Concentrations of CMT 308 up to 50 μM caused no cytotoxicity to MCF-7 or MDA-MB-435s (Fig 3) cells over a 24h period. Levels of mitochondrial dehydrogenase activity remained stable at all concentrations of CMT-308 up to levels comparable to the concentrations of CMT-300 that have been reported to be reached in the circulation of human subjects or animal models after oral administration. Thus, the reduction in VEGF secretion with CMT 308 treatment was not a result of reduced cell numbers due to cytotoxicity.

**Time course of VEGF inhibition by CMT 308 in MCF-7 cells**

To evaluate whether inhibition of VEGF secretion from MCF-7 cells by CMT 308 occurs concurrently with the time of appearance of newly secreted protein, VEGF levels were measured in the conditioned medium of MCF-7 cells treated with 20 μM CMT 308 for various times. Basal VEGF production in cultures of cells that were not stimulated by a growth factor was detectable 4 hours after plating the cells in serum-free media, and rose progressively over the next 24 hours (Fig 4). In the presence of 10 ng/ml TGFβ, newly secreted VEGF could be detected in the culture medium as early as 2 hours after plating the MCF-7 cells. CMT 308 (20 μM) inhibited basal VEGF secretion at the earliest time points that the angiogenic factor could be detected in the medium of untreated control cells, and VEGF levels in the medium of CMT 308-treated cells remained undetectable up to 8 hours. In the medium from MCF-7 cells that were simultaneously treated with 10 ng/ml TGFβ and 20 μM CMT 308, no VEGF could be detected up to 8h after addition of the growth factor and the tetracycline to the culture medium. During the interval between 8 and 24h after addition of the two agents, low levels of VEGF began to accumulate in the conditioned medium, indicating that the inhibitory effect of CMT 308 on VEGF release was diminished after 8 hours regardless of the presence or absence of TGFβ (Fig 4).

**CMT 308 diminishes secretion of VEGF from MCF-7 cells for up to 8 hours, regardless of previous time in culture**

To characterize more completely the duration of efficacy of CMT 308 in inhibiting release of VEGF, MCF-7 cells were treated with 20 μM CMT 308 either immediately after plating, or 8 hours after plating. In cells that were treated at the onset with CMT 308, VEGF secretion remained undetectable over the 8h duration. Thereafter, a gradual increase in VEGF levels in the conditioned medium was observed. (Fig 5) In the medium from cells that were treated with CMT 308 after 8h of plating, the levels of VEGF that had accumulated for the first 8h in the absence of the tetracycline did not subsequently fall, but no additional secreted VEGF was detected over the next 8 hours. Thus, the inhibitory effects of CMT 308 lasted up to 8h regardless of the time at which the cells were treated with the CMT. This confirmed the ability of the CMT to inhibit release of newly secreted VEGF, while having little or no effect on the levels of VEGF protein which had already been released into the conditioned medium. The apparent loss of efficacy of CMT-308 in inhibiting release of VEGF over time may be due to some catabolism of the CMT by the cells, or alternatively may reflect enhanced efflux of the CMT from the cell.

**CMTs do not modulate steady state VEGF mRNA levels in breast cancer cell lines**

In order to determine whether the effect of CMT-308 on diminution in levels of VEGF released by MCF-7 and MDA-MB-435s cells was due to reduction in VEGF mRNA levels, steady state mRNA levels in both the cells lines were examined after treatment with CMTs and TGFβ using RT-PCR. Primers were chosen to distinguish between the transcripts for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ isoforms. In MCF-7 as well as MDA-MB-435s, the only transcripts detectable were for the secreted isoforms - $VEGF_{121}$ and $VEGF_{165}$. Neither of the mRNAs for the largely intracellular 189 amino acid nor the 145 amino acid ECM-sequestered isoform of VEGF was detected in either cell line.

Although dose-dependent reductions in levels of secreted VEGF protein were observed after treatment of cells with CMT 308, concentrations of this tetracycline derivative from 1 μM to 50 μM did not result in reduced VEGF mRNA levels in MCF-7 cells (Fig 6). Additionally, TGFβ at 1 ng/ml failed to induce increased VEGF mRNA levels in MCF-7 cells although it induced augmented secretion of VEGF into the conditioned medium (Fig 6). VEGF mRNA levels in MCF-7 cells treated with 5 μM CMT 300 or 20 μM CMT 308 for 6 hours were unaffected regardless of pre-treatment with TGFβ (Fig 7A). This was also confirmed by a quantitative assay for VEGF mRNA (Fig 7C). As noted, levels of VEGF mRNA relative to GAPDH mRNA did not change significantly in MCF-7 cells treated with CMT 308.

VEGF mRNA levels of the more invasive MDA-MB-435s cells also remained unaffected by TGFβ treatment, in contrast to the augmented levels of VEGF protein in the surrounding medium from the cells (Fig 7B). Similar to the observations with MCF-7 cells, neither 5 μM CMT 300 nor 20 μM CMT 308 reduced VEGF mRNA levels in TGFβ treated or untreated cells (Fig 7B). The results indicated that CMT 308 did not reduce the levels of VEGF protein secreted by these cell lines by modulating steady-state levels of the mRNA for VEGF.

**CMTs modulate intracellular VEGF levels in the same manner as the secreted VEGF levels in breast cancer cell lines**

To determine whether CMTs diminish secretion of VEGF by modulating intracellular levels of VEGF, lysates of MDA-MB-435s cells treated with the CMTs were evaluated for VEGF protein by ELISA. As expected for a secreted cytokine, total steady-state intracellular VEGF levels were significantly lower (Fig 8, Left Y axis) than secreted VEGF levels which accumulated in the conditioned media (Fig 8 Right Y axis). Treatment with 1 ng/ml TGFβ significantly increased the intracellular VEGF pool similar to this growth factor's effect on increasing levels of secreted VEGF (Fig 8). Treatment with CMT 300 also increased intracellular VEGF levels, reflecting the pattern observed

with secreted VEGF levels. Interestingly, treatment with CMT 308 did not significantly reduce intracellular VEGF pools in cells that were not treated with TGFβ; instead, VEGF levels were similar to untreated cells. However, in cells treated with TGFβ, addition of CMT 308 reduced intracellular VEGF levels analogous to the reduction seen in the secreted levels. Additionally, total protein levels in cell lysates and conditioned media were not modulated by treatment with the CMTs (data not shown). Hence, the effects of the CMTs on VEGF are not due to a general reduction in total protein levels. The effect of CMTs on VEGF levels in cell lysates of TGFβ-treated cells thus parallels their effect on extracellular VEGF levels.

## DISCUSSION

In this study, we demonstrate a novel activity of a new CMT, CMT 308 against a pro-angiogenic molecule that may contribute to its anti-angiogenic action independent of its anti-metalloprotease activity. Our studies demonstrate the capacity of CMTs to influence the angiogenic potential of breast cancer cell lines *in vitro* and provide some insights into the possible mode of action of these non-antimicrobial tetracycline derivatives. We used these two different cell lines as representatives of different stages of breast cancer. The MCF-7 cell line, derived from pleural effusion of a breast adenocarcinoma, is a poorly invasive, estrogen-responsive cell line with low tumorigenicity (31). The MDA-MB-435s cell line, derived from a metastatic ductal carcinoma, is a highly invasive, estrogen-unresponsive cell line with high tumorigenic capacity (31). We observed that in the absence of growth factors, MDA-MB-435s cells release significantly higher levels of VEGF than MCF-7 cells. This is consistent with the characterizations of this cell line as a model for late stage breast cancer. Furthermore, higher levels of VEGF have been noted in the sera of patients with estrogen receptor-negative tumors than in patients with estrogen receptor-positive tumors (32), validating the use of these two cell lines as models for different stages of tumor progression. Our results show that CMT 308 effectively reduced basal VEGF secretion in these breast cancer cell lines without accompanying cytotoxicity, reflecting an activity of this derivative on some step in a constitutive pathway for VEGF secretion in these cells.

Our observation that TGFβ increases VEGF secretion in the breast cancer cell lines is consistent with its role as an indirect inducer of angiogenesis. CMT 308 reduced VEGF secretion in cells stimulated with TGFβ down to basal levels, perhaps by affecting TGFβ-stimulated pathways. Precisely which pathway regulating VEGF production is responsive to TGFβ in these tumor cell lines is not clear. TGFβ appears to act via different pathways in different cell types such as the Ras pathway (33, 34), AP-1/HIF-1α pathway (35, 36) or the p38 MAP Kinase pathway (37, 38). Our experiments show that CMT 308 affects one or more of the pathways through which TGFβ can induce augmented VEGF secretion by the tumor cell lines. However, because TGFβ-

induced VEGF secretion is not completely suppressed by CMT 308, VEGF production may be controlled through CMT-sensitive as well as CMT-insensitive pathways. Elucidation of the specific mechanism of action of CMT 308 and its targets will involve the dissection of these known pathways or identification of other, as yet unknown pathways.

Unlike CMT 308, CMT 300, which lacks an amino group on the 9-position of the tetracycline ring system but is otherwise identical in chemical structure to CMT-308, has a modest stimulatory effect on basal levels of VEGF released by MCF-7 cells. CMT 300 has an even more marked dose-dependent stimulatory effect on both, basal and TGFβ-induced VEGF that is cytoplasmic as well as secreted in MDA-MB-435s cells. CMT 300 is known to be more cytotoxic than CMT 308, induces apoptosis more efficiently than CMT 308, and can more efficiently collapse the mitochondrial membrane potential in prostate cancer cells (14).

The time course of CMT 308's effect on VEGF in MCF-7 cells indicates that the apparent potency of CMT 308 as an inhibitor of VEGF secretion in breast cancer cells is a function of inhibitor dose as well as time in the presence of the inhibitory agent. MCF-7 cells had an apparent capacity to escape from the suppressive effects of CMT 308 after 8 hours of exposure. The resumption of VEGF secretion seen after 8 hours may be due to progressive loss of sensitivity of one or more of the VEGF secretory pathways to inhibition by CMT 308. It may also be a result of a time-dependent capacity of the cells to clear the drug. It is unlikely, however that the drug itself loses potency, since in monocytic cells CMT 308 remains a potent inhibitor of VEGF secretion up to 48 hours (data not shown). Addition of CMT 308 after 8 hours blocks any further increases in the levels of VEGF in the conditioned medium from MCF-7 cells up to an additional 8 hours, but the pre-existing VEGF levels do not decline, indicating that new secretion of VEGF may be inhibited by CMT 308, while pre-existing VEGF protein in the conditioned medium is not affected by the addition of CMT 308.

While Seftor et al have observed that CMT 300 decreased mRNA levels for VEGF-C in human cutaneous and uveal melanoma cell lines (39), in the breast cancer cell lines that we used, the reduction in secreted VEGF produced by CMT 308 was not a result of inhibition of transcription of the $VEGF_{121}$ and $VEGF_{165}$ mRNA isoforms. The variations in the VEGF mRNA species isolated and in the cell lines used may account for these apparently contradictory results. We also did not observe changes in the concentrations of the different VEGF mRNA species upon treatment of cells with TGFβ, although TGFβ has been shown to increase VEGF mRNA levels in several other cell types such as osteoblasts (9), fibroblasts (10) and keratinocytes (40) and other breast tumor cells (11) by inducing transcription of the VEGF gene. Although the levels of VEGF mRNAs were unaltered in MCF-7 and MDA-MB-435s cells upon addition of TGFβ, we did observe enhanced secretion of VEGF by both cell lines in the presence of the growth factor. Significantly, there was no effect of CMT 300 or CMT 308 on VEGF mRNA levels in these TGFβ-stimulated cells. Our results are consistent with the report that CMT 300 does not inhibit transcription of the inducible Nitric Oxide Synthase (iNOS) gene in murine macrophages (41, 42), nor does it affect COX-2 mRNA in murine macrophages (43). CMT 300 does, however reduce post-transcriptional stability of iNOS mRNA in the murine macrophages (41). We observed that neither CMT 300 nor CMT 308 affected alternative splicing of VEGF mRNA in the breast cancer cell lines we examined, an event that occurs post-transcriptionally.

The reduction in levels of intracellular pools of VEGF, but not of VEGF mRNA levels in breast cancer cells treated with CMT 308 indicates that CMT 308 may affect post-transcriptional events in these cells. CMTs may affect cytoplasmic accumulation of VEGF by inhibiting translation of VEGF protein without affecting transcription. Alternatively, CMTs may degrade VEGF protein upon translation. Although CMT 308 does not cause a generalized reduction in total protein levels in these cells, specific degradation of cytoplasmic VEGF may be possible, perhaps by targeted degradation through the ubiquitin-proteasome pathway. Additionally, it is possible that CMT 308 induces a redistribution of intracellular VEGF into different compartments such as the ER where glycoslylation of VEGF and folding take place (44). Since proteins that are

mis-folded are often retained in the ER, it may be possible that CMT 308 causes a post-translational mis-folding of protein as suggested by Patel et al (43). Mis-folded proteins are often targeted for degradation via the ubiquitin pathway, leading to reduction in steady-state levels of the specific protein. The relatively low cytoplasmic levels compared to extracellular levels indicate that the VEGF protein is predominantly secreted rather than stored. Treatment with TGFβ increases cytoplasmic VEGF as well as secretion of VEGF, implying that TGFβ may modulate translation of the VEGF protein and its subsequent secretion.

The possibility that the reduction of VEGF in the conditioned medium may be a result of CMT 308's metalloprotease inhibitory activity must be considered. It has been observed that *in vivo,* matrix metalloprotease-9 (MMP-9) can trigger the angiogenic switch by increasing the availability of VEGF in very early stages of pancreatic tumors (45). If inhibition of MMP9 were the main cause for the reduction in VEGF secretion however, then comparable diminutions in VEGF would have been noted after treatment with CMT 300 and CMT 308, which are both potent inhibitors of MMP9 (46, 47). Also, human glioma cells that are transfected with membrane-type I MMP (MT-I MMP) secrete greater levels of VEGF in the conditioned media than control cells (48). This increase in secreted VEGF can be down-regulated by inhibitors of MT-I-MMP such as GM6001, a general MMP inhibitor. CMT 300 has been shown to inhibit MT-I MMP activation of pro-MMP-2 in COS-1 cells (49). However, our results with MCF-7 and MDA-MB-435s cells show that CMT 300 is not an effective inhibitor of VEGF release. This makes it unlikely that the diminished levels of secreted VEGF in cells treated with CMT 308 (which has similar MMP-inhibitory activity as CMT 300) can be attributed to inhibition of known metalloprotease activities. More specifically, the inhibition of VEGF release mediated by CMT-308 does not appear to be a result of inhibition of soluble MMPs or MT-I MMP.

VEGF is an attractive target for anti-angiogenic therapy in breast cancer because VEGF production is known to be high in many human breast cancers (50), the vascular density of tumors has been correlated with metastasis and outcome in patients

with breast cancer (51-53), and patients with high tumor VEGF levels have the poorest prognosis and survival (54-56). As a result of these observations, various strategies to inhibit angiogenesis are being developed (57-59). The novel mechanism of action of CMT 308 compared to the currently available strategy utilizing neutralizing antibody to VEGF makes CMT 308 an attractive therapeutic candidate. The dual ability of CMT 308 to inhibit basal VEGF secretion as well as TGFβ mediated secretion in breast tumor cell lines in the absence of significant cytotoxicity suggests that it may be useful in the management of breast cancer in combination therapy with other more traditional chemotherapeutic agents.

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## References


1. Folkman, J Tumor angiogenesis: therapeutic implications. N Engl J Med, 1971; *285*(21)*:* 1182-1186.
2. Gerwins, P, Skoldenberg, E, and Claesson-Welsh, L Function of fibroblast growth factors and vascular endothelial growth factors and their receptors in angiogenesis. Crit Rev Oncol Hematol, 2000; *34*(3)*:* 185-194.
3. Relf, M, LeJeune, S, Scott, PA*, et al.* Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res, 1997; *57*(5)*:* 963-969.
4. Carmeliet, P, Dor, Y, Herbert, JM*, et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature, 1998; *394*(6692)*:* 485-490.
5. Fukumura, D, Xavier, R, Sugiura, T*, et al.* Tumor induction of VEGF promoter activity in stromal cells. Cell, 1998; *94*(6)*:* 715-725.
6. Ruohola, JK, Valve, EM, Karkkainen, MJ, Joukov, V, Alitalo, K, and Harkonen, PL Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol, 1999; *149*(1-2)*:* 29-40.
7. Pepper, MS Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev, 1997; *8*(1)*:* 21-43.
8. Schwarte-Waldhoff, I, Volpert, OV, Bouck, NP*, et al.* Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc Natl Acad Sci U S A, 2000; *97*(17)*:* 9624-9629.
9. Saadeh, PB, Mehrara, BJ, Steinbrech, DS*, et al.* Transforming growth factor-beta1 modulates the expression of vascular endothelial growth factor by osteoblasts. Am J Physiol, 1999; *277*(4 Pt 1)*:* C628-637.
10. Pertovaara, L, Kaipainen, A, Mustonen, T*, et al.* Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem, 1994; *269*(9)*:* 6271-6274.
11. Donovan, D, Harmey, JH, Toomey, D, Osborne, DH, Redmond, HP, and Bouchier-Hayes, DJ TGF beta-1 regulation of VEGF production by breast cancer cells. Ann Surg Oncol, 1997; *4*(8)*:* 621-627.
12. Golub, LM, Schuler, EK, and Gallagher, BM New therapeutic uses for an old family of drugs: travels of a dental researcher from the lab to the university's office of technology transfer and beyond. Technol Health Care, 1996; *4*(3)*:* 311-316.
13. Meng, Q, Xu, J, Goldberg, ID, Rosen, EM, Greenwald, RA, and Fan, S Influence of chemically modified tetracyclines on proliferation, invasion and migration properties of MDA-MB-468 human breast cancer cells. Clin Exp Metastasis, 2000; *18*(2)*:* 139-146.
14. Lokeshwar, BL, Escatel, E, and Zhu, B Cytotoxic activity and inhibition of tumor cell invasion by derivatives of a chemically modified tetracycline CMT-3 (COL-3). Curr Med Chem, 2001; *8*(3)*:* 271-279.

15. Lokeshwar, BL, Selzer, MG, Zhu, BQ, Block, NL, and Golub, LM Inhibition of cell proliferation, invasion, tumor growth and metastasis by an oral non-antimicrobial tetracycline analog (COL-3) in a metastatic prostate cancer model. Int J Cancer, 2002; *98*(2)*:* 297-309.
16. Lokeshwar, BL, Houston-Clark, HL, Selzer, MG, Block, NL, and Golub, LM Potential application of a chemically modified non-antimicrobial tetracycline (CMT-3) against metastatic prostate cancer. Adv Dent Res, 1998; *12*(2)*:* 97-102.
17. Lukkonen, A, Sorsa, T, Salo, T*, et al.* Down-regulation of trypsinogen-2 expression by chemically modified tetracyclines: association with reduced cancer cell migration [In Process Citation]. Int J Cancer, 2000; *86*(4)*:* 577-581.
18. Gu, Y, Musacchia, L, Simon, S, Roemer, E, Lee, H, and Golub, L Inhibition of tumor cell invasiveness by chemically modified tetracyclines. Curr Med Chem, 2001; *8*(3)*:* 261-270.
19. Seftor, RE, Seftor, EA, De Larco, JE*, et al.* Chemically modified tetracyclines inhibit human melanoma cell invasion and metastasis. Clin Exp Metastasis, 1998; *16*(3)*:* 217-225.
20. D'Agostino, P, Ferlazzo, V, Milano, S*, et al.* Chemically modified tetracyclines induce cytotoxic effects against J774 tumour cell line by activating the apoptotic pathway. Int Immunopharmacol, 2003; *3*(1)*:* 63-73.
21. Tolomeo, M, Grimaudo, S, Milano, S*, et al.* Effects of chemically modified tetracyclines (CMTs) in sensitive, multidrug resistant and apoptosis resistant leukaemia cell lines. Br J Pharmacol, 2001; *133*(2)*:* 306-314.
22. Fife, RS, Sledge, GW, Jr., Sissons, S, and Zerler, B Effects of tetracyclines on angiogenesis in vitro. Cancer Lett, 2000; *153*(1-2)*:* 75-78.
23. Gilbertson-Beadling, S, Powers, EA, Stamp-Cole, M*, et al.* The tetracycline analogs minocycline and doxycycline inhibit angiogenesis in vitro by a non-metalloproteinase-dependent mechanism. Cancer Chemother Pharmacol, 1995; *36*(5)*:* 418-424.
24. Hanemaaijer, R, Visser, H, Koolwijk, P*, et al.* Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. Adv Dent Res, 1998; *12*(2)*:* 114-118.
25. Nakamura, S, Murakami-Mori, K, Rao, N, Weich, HA, and Rajeev, B Vascular endothelial growth factor is a potent angiogenic factor in AIDS-associated Kaposi's sarcoma-derived spindle cells. J Immunol, 1997; *158*(10)*:* 4992-5001.
26. Cianfrocca, M, Cooley, TP, Lee, JY*, et al.* Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS malignancy consortium study. J Clin Oncol, 2002; *20*(1)*:* 153-159.
27. Pantanowitz, L, Dezube, BJ, Pinkus, GS, and Tahan, SR Histological characterization of regression in acquired immunodeficiency syndrome-related Kaposi's sarcoma. J Cutan Pathol, 2004; *31*(1)*:* 26-34.
28. Munaut, C, Noel, A, Hougrand, O, Foidart, JM, Boniver, J, and Deprez, M Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. Int J Cancer, 2003; *106*(6)*:* 848-855.

29. Kroon, AM, Dontje, BH, Holtrop, M, and Van den Bogert, C The mitochondrial genetic system as a target for chemotherapy: tetracyclines as cytostatics. Cancer Lett, 1984; *25*(1)*:* 33-40.
30. van den Bogert, C, Dontje, BH, and Kroon, AM The antitumour effect of doxycycline on a T-cell leukaemia in the rat. Leuk Res, 1985; *9*(5)*:* 617-623.
31. Zajchowski, DA, Bartholdi, MF, Gong, Y*, et al.* Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res, 2001; *61*(13)*:* 5168-5178.
32. Heer, K, Kumar, H, Read, JR, Fox, JN, Monson, JR, and Kerin, MJ Serum vascular endothelial growth factor in breast cancer: its relation with cancer type and estrogen receptor status. Clin Cancer Res, 2001; *7*(11)*:* 3491-3494.
33. Breier, G, Blum, S, Peli, J*, et al.* Transforming growth factor-beta and Ras regulate the VEGF/VEGF-receptor system during tumor angiogenesis. Int J Cancer, 2002; *97*(2)*:* 142-148.
34. Janda, E, Lehmann, K, Killisch, I*, et al.* Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol, 2002; *156*(2)*:* 299-313.
35. Shih, SC and Claffey, KP Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. Growth Factors, 2001; *19*(1)*:* 19-34.
36. Sanchez-Elsner, T, Botella, LM, Velasco, B, Corbi, A, Attisano, L, and Bernabeu, C Synergistic cooperation between hypoxia and transforming growth factor- beta pathways on human vascular endothelial growth factor gene expression. J Biol Chem, 2001; *276*(42)*:* 38527-38535.
37. Yamamoto, T, Kozawa, O, Tanabe, K*, et al.* Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. J Cell Biochem, 2001; *82*(4)*:* 591-598.
38. Goldberg, PL, MacNaughton, DE, Clements, RT, Minnear, FL, and Vincent, PA p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. Am J Physiol Lung Cell Mol Physiol, 2002; *282*(1)*:* L146-154.
39. Seftor, RE, Seftor, EA, Kirschmann, DA, and Hendrix, MJ Targeting the tumor microenvironment with chemically modified tetracyclines: inhibition of laminin 5 gamma2 chain promigratory fragments and vasculogenic mimicry. Mol Cancer Ther, 2002; *1*(13)*:* 1173-1179.
40. Frank, S, Hubner, G, Breier, G, Longaker, MT, Greenhalgh, DG, and Werner, S Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem, 1995; *270*(21)*:* 12607-12613.
41. Amin, AR, Patel, RN, Thakker, GD, Lowenstein, CJ, Attur, MG, and Abramson, SB Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines. FEBS Lett, 1997; *410*(2-3)*:* 259-264.
42. Trachtman, H, Futterweit, S, Greenwald, R*, et al.* Chemically modified tetracyclines inhibit inducible nitric oxide synthase expression and nitric oxide production in cultured rat mesangial cells. Biochem Biophys Res Commun, 1996; *229*(1)*:* 243-248.

43. Patel, RN, Attur, MG, Dave, MN*, et al.* A novel mechanism of action of chemically modified tetracyclines: inhibition of COX-2-mediated prostaglandin E2 production. J Immunol, 1999; *163*(6)*:* 3459-3467.
44. Vega, VL and De Maio, A Geldanamycin treatment ameliorates the response to LPS in murine macrophages by decreasing CD14 surface expression. Mol Biol Cell, 2003; *14*(2)*:* 764-773.
45. Bergers, G, Brekken, R, McMahon, G*, et al.* Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol, 2000; *2*(10)*:* 737-744.
46. Greenwald, RA, Golub, LM, Ramamurthy, NS, Chowdhury, M, Moak, SA, and Sorsa, T In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. Bone, 1998; *22*(1)*:* 33-38.
47. Ramamurthy, NS, Rifkin, BR, Greenwald, RA*, et al.* Inhibition of matrix metalloproteinase-mediated periodontal bone loss in rats: a comparison of 6 chemically modified tetracyclines. J Periodontol, 2002; *73*(7)*:* 726-734.
48. Deryugina, EI, Soroceanu, L, and Strongin, AY Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis. Cancer Res, 2002; *62*(2)*:* 580-588.
49. Lee, HM, Golub, LM, Cao, J*, et al.* CMT-3, a non-antimicrobial tetracycline (TC), inhibits MT1-MMP activity: relevance to cancer. Curr Med Chem, 2001; *8*(3)*:* 257-260.
50. Toi, M, Kondo, S, Suzuki, H*, et al.* Quantitative analysis of vascular endothelial growth factor in primary breast cancer. Cancer, 1996; *77*(6)*:* 1101-1106.
51. Weidner, N, Semple, JP, Welch, WR, and Folkman, J Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med, 1991; *324*(1)*:* 1-8.
52. Hollingsworth, HC, Kohn, EC, Steinberg, SM, Rothenberg, ML, and Merino, MJ Tumor angiogenesis in advanced stage ovarian carcinoma. Am J Pathol, 1995; *147*(1)*:* 33-41.
53. Ellis, LM and Fidler, IJ Angiogenesis and breast cancer metastasis. Lancet, 1995; *346*(8972)*:* 388-390.
54. Foekens, JA, Peters, HA, Grebenchtchikov, N*, et al.* High tumor levels of vascular endothelial growth factor predict poor response to systemic therapy in advanced breast cancer. Cancer Res, 2001; *61*(14)*:* 5407-5414.
55. Gasparini, G Prognostic value of vascular endothelial growth factor in breast cancer. Oncologist, 2000; *5*(Suppl 1)*:* 37-44.
56. Gasparini, G and Harris, AL Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. J Clin Oncol, 1995; *13*(3)*:* 765-782.
57. Folkman, J, Browder, T, and Palmblad, J Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost, 2001; *86*(1)*:* 23-33.
58. Rosen, L Antiangiogenic strategies and agents in clinical trials. Oncologist, 2000; *5*(Suppl 1)*:* 20-27.
59. Saijo, N, Tamura, T, and Nishio, K Strategy for the development of novel anticancer drugs. Cancer Chemother Pharmacol, 2003; *52 Suppl 1*(S97-101.

## Figure Legends

**Fig 1: CMT 308 inhibits basal and TGFβ-stimulated VEGF secretion from MCF-7 and MDA-MB-435s cells.** **A.** MCF-7 cells or **B.** MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium. Cells were treated with 0.1, 1 or 10 ng/ml TGFβ. In addition, the cells received either diluted DMSO vehicle alone (black bars), 20 μM (clear bar) CMT 300 or 20 μM (hatched bar) CMT 308. Cells were incubated for a further 24h at $37^0$C. Conditioned medium was assayed for VEGF by ELISA. **A.** TGFβ from 0.1 ng/ml to 10 ng/ml significantly (*$p<0.05$) stimulated VEGF secretion from MCF-7 cells. CMT 308 significantly (*$p<0.05$) reduced VEGF secretion relative to cells teated with vehicle alone. CMT 300 at 20 μM did not significantly affect VEGF secretion. **B.** TGFβ at 1 and 10 ng/ml significantly (***$p<0.0001$) stimulated VEGF secretion from MDA-MB-435s cells. CMT 308 significantly decreased basal (***$p<0.0001$) and TGFβ-stimulated VEGF secretion at levels of growth factor of 0.1 ng/ml and 1 ng/ml (***$p<0.0001$) as well as at 10 ng/ml (**$p<0.01$). CMT 300 at 20 μM also did not significantly affect VEGF secretion except in TGFβ-untreated cells (*$p<0.05$), Results represent at least three independent experiments ± SEM.

**Fig 2: CMT 308 reduces TGFβ-induced VEGF secretion from MCF-7 and MDA-MB-435s cells in a dose-dependent manner. A.** MCF-7 or **B.** MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium. A. MCF-7 cells were treated with diluted DMSO vehicle alone or 1 to 50 μM CMT 308 in addition to 1 ng/ml TGFβ as indicated and **B.** MDA-MB-435s cells were treated with 1 to 50 μM CMT 300 (black bars) or CMT 308 (clear bars). Cells were incubated for a further 24h at $37^0$C. Conditioned medium was then assayed for VEGF by ELISA. A. CMT 308 caused a significant ($p<0.0001$) dose-dependent reduction in VEGF secretion from MCF-7 cells. **B.** CMT 308 at 20 μM significantly (*$p<0.05$) reduced VEGF secretion while at 30 and 50 μM the reduction was very significant (**$p<0.005$). CMT 300 did not significantly affect VEGF secretion. Results represent at least three independent experiments ± SEM.

**Fig 3: CMTs are minimally cytotoxic to MCF-7 and MDA-MB-435s cells.** MCF-7 or MDA-MB-435s cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated with 5, 10, 20, or 30 μM CMT 300 or CMT 308. Serum-free medium was added to the cells after CMT-treatment and MTS solution added for up to 4 hours, and absorbance of the formazan product generated by viable cells read at 490 nm. Percent viable cells were calculated relative to untreated cells. Neither CMT 300 in MCF-7 cells (-■-) or in MDA-MB-435s (-▲-) nor CMT 308 in MCF-7 (-□-) or in MDA-MB-435s (-△-) was cytotoxic to up to 30 μM. Results represent at least three independent experiments ± SEM.

**Fig 4: Time course of the effect of CMTs on VEGF secretion by MCF-7 cells.** MCF-7 cells were plated in complete medium overnight at $37^0$C. Medium was then replaced with serum-free medium and cells were treated either with diluted DMSO vehicle alone (black bars) or with 20 μM CMT 308 (grey bar), or with 10 ng/ml TGFβ (hatched bar) as well as 20 μM CMT 308 (spotted bar). At specific times after treatment as indicated, conditioned medium was collected and assayed for VEGF by ELISA. Results represent at least three independent experiments ± SEM.

**Fig 5: CMT 308 is effective for up to 8 hours of treatment in MCF-7 cells regardless of time of treatment.** MCF-7 cells were plated in complete medium and incubated overnight at $37^0$C. Next day, medium was replaced with serum-free medium. One set of cells was left untreated (black bars) and conditioned medium was collected from these cells immediately after plating at 0h (Bar A), 8h after plating (Bar B) or 16h after plating (Bar c). A second set of cells was treated with 20 μM CMT 308 at the time of plating at 0h (clear bars). Conditioned medium was collected from these cells immediately upon plating (Bar D), 8h after plating (Bar E) or 16h after plating (Bar F). A third set of cells was treated with CMT 308 8h after plating (hatched bars). Conditioned media from these cells was collected immediately, that is, 8h after plating but 0h after adding CMT 308 (Bar G) or 16h after plating but 8h after adding CMT 308 (Bar H). Untreated cells produced significant (***$p<0.0001$) amounts of VEGF 8h and 16 h after plating (Bars A, B, C). CMT 308 significantly (***$p<0.0001$) reduced VEGF secretion up to 8h (Bar E) after plating compared to the untreated control (Bar D). As the effects of CMT 308 diminished after 8h of treatment, cells produced significant amounts (***$p<0.0001$) of VEGF at 16h (Bar F) compared to the levels of VEGF in cultures maintained for 8h after treatment (Bar E). In addition, when cells were not treated with CMT 308 until 8h after plating (Bar G), the accumulated VEGF levels did not significantly increase further over the next 8h. (Bar H). Results represent three independent experiments ± SEM.

**Fig 6: CMT 308 at subcytotoxic doses does not reduce VEGF mRNA levels in MCF-7 cells.** MCF-7 cells were treated with 1 ng/ml TGFβ and varying doses of CMT 308 for 24h. Equal quantities of total RNA were assayed using PCR. VEGF primers were chosen to distinguish between $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, and $VEGF_{121}$. The only VEGF mRNA species detectable were $VEGF_{165}$ and $VEGF_{121}$. TGFβ did not increase VEGF mRNA levels (Lanes 1 and 2). Treatment with CMT 308 from 1 μM to 50 μM (Lanes 3-7) did not decrease VEGF mRNA levels in MCF-7 cells. Results represent at least three independent experiments.

**Fig 7: TGFβ does not increase VEGF mRNA and CMT 308 does not reduce VEGF mRNA levels in (A) MCF-7 or (B) MDA-MB-435s cells. A.** MCF-7 cells **B.** or MDA-MB-435s cells were treated with 5 μM CMT 300 or 20 μM CMT 308 in the absence or presence of TGFβ for 6h. Equal quantities of total RNA were assayed using RT-PCR. The only VEGF mRNA species detectable were $VEGF_{165}$ and $VEGF_{121}$. Treatment with 1 ng/ml TGFβ did not cause an increase in VEGF mRNA levels (Lanes 1 & 4) in either MCF-7 (A) or MDA-MB-435s (B) cells. Neither addition of CMT 300 (5 μM) nor CMT 308 (20 μM) reduced VEGF mRNA in cells cultured in the presence (Lanes 5 & 6) or absence of TGFβ (Lanes 2 & 3) in either A or B. **C.** Quantitation of VEGF mRNA in MCF-7 cells relative to GAPDH mRNA levels using Quantikine ELISA method for analysis. Results represent at least three independent experiments.

**Fig 8: TGFβ increases intracellular VEGF levels and CMT 308 reduces intracellular VEGF levels in MDA-MB-435s cells.** MDA-MB-435s cells were cultured either in the presence or the absence of 1 ng/ml TGFβ and were treated with 20 μM CMT 300 or 20 μM CMT 308 for 24h. Conditioned media and cell lysates were collected and analyzed for VEGF with ELISA. VEGF levels shown have been corrected for total volume of cell lysates (100 uL) and conditioned media (10 ml),.Cell lysate levels (black bars) are represented on the Left Y axis, while the levels in conditioned media (clear bars) are represented on the Right Y axis. Results represent two independent experiments.

**Fig 1A**

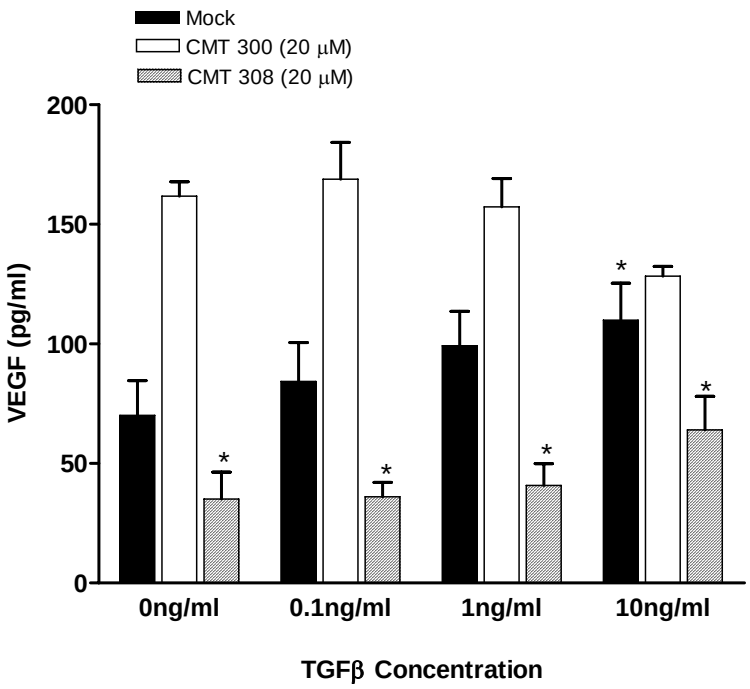

Mock
CMT 300 (20 μM)
CMT 308 (20 μM)
VEGF (pg/ml)
200
150
100
50
0
*
*
*
*
*
0ng/ml
0.1ng/ml
1ng/ml
10ng/ml
TGFβ Concentration

**Fig 1B**

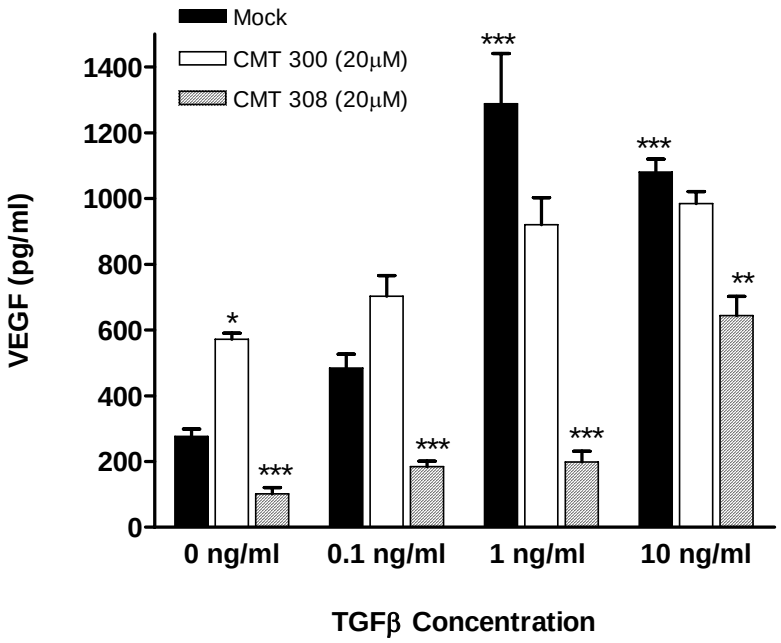

Mock
CMT 300 (20μM)
CMT 308 (20μM)
VEGF (pg/ml)
1400
1200
1000
800
600
400
200
0
*
***
***
***
***
**
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ Concentration

**Fig 2A**

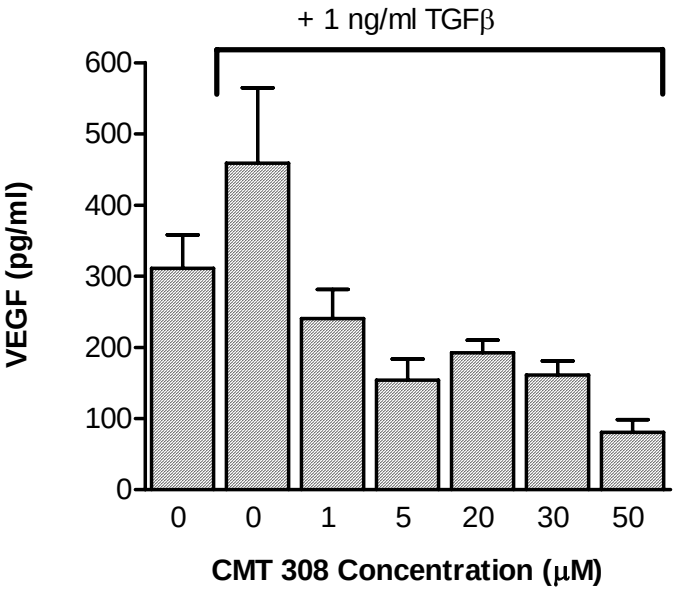

+ 1 ng/ml TGFβ
600
500
400
300
200
100
0
VEGF (pg/ml)
0
0
1
5
20
30
50
CMT 308 Concentration (μM)

**Fig 2B**

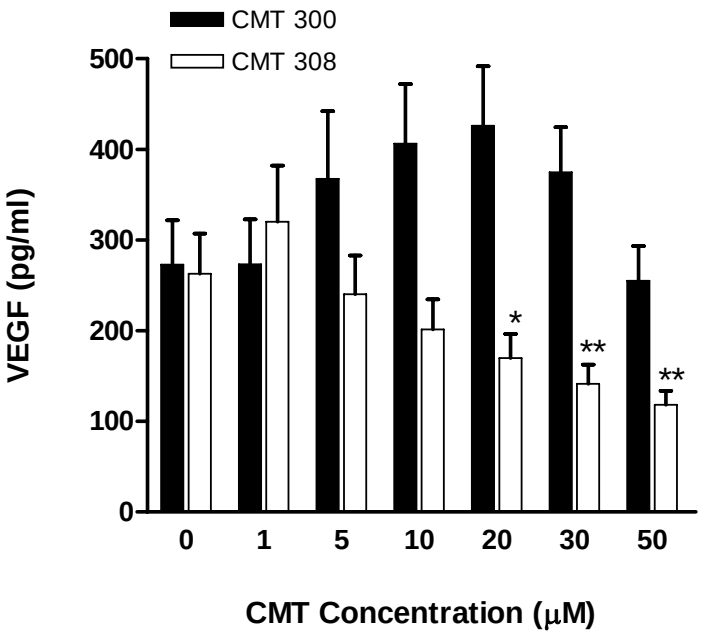

CMT 300
CMT 308
VEGF (pg/ml)
500
400
300
200
100
0
*
**
**
0
1
5
10
20
30
50
CMT Concentration (μM)

**Fig 3**

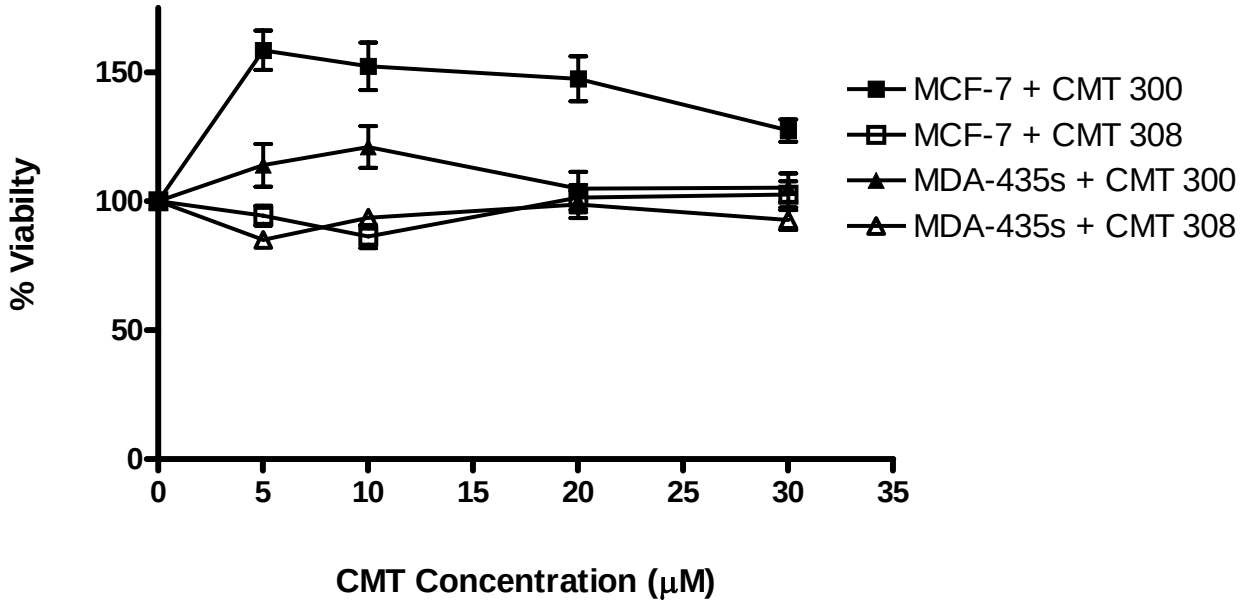

% Viability
150
100
50
0
0
5
10
15
20
25
30
35
CMT Concentration (μM)
MCF-7 + CMT 300
MCF-7 + CMT 308
MDA-435s + CMT 300
MDA-435s + CMT 308

**Fig 4**

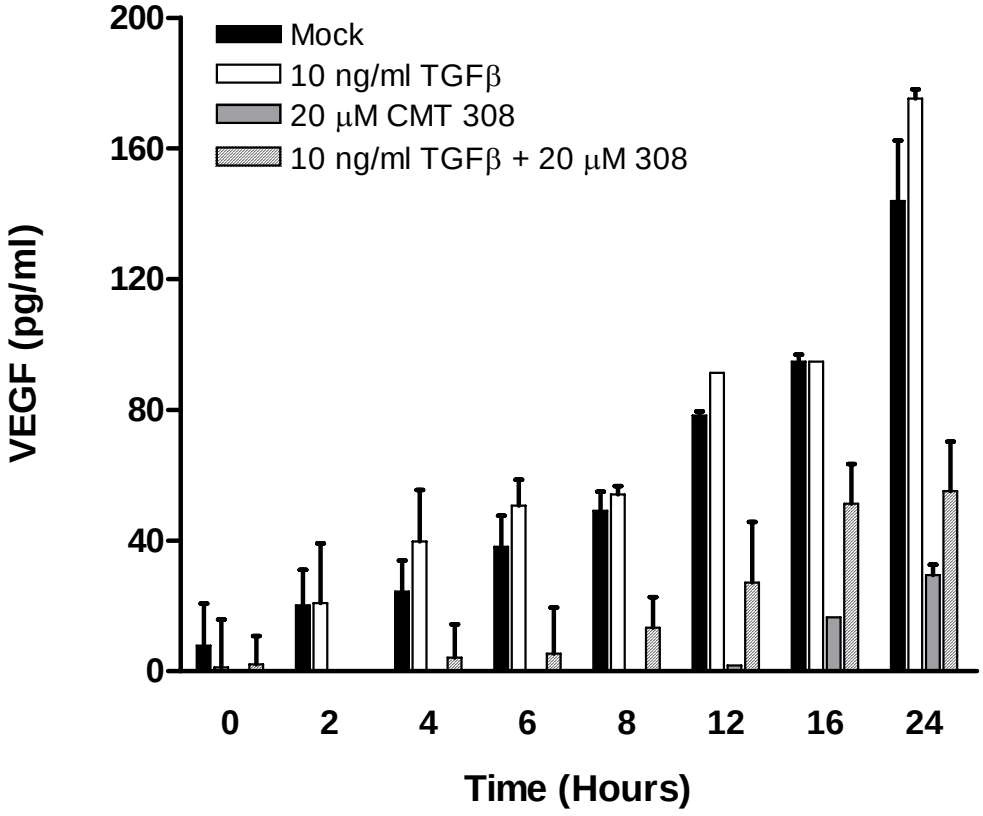

Mock
10 ng/ml TGFβ
20 μM CMT 308
10 ng/ml TGFβ + 20 μM 308
VEGF (pg/ml)
200
160
120
80
40
0
0
2
4
6
8
12
16
24
Time (Hours)

**Fig 5**

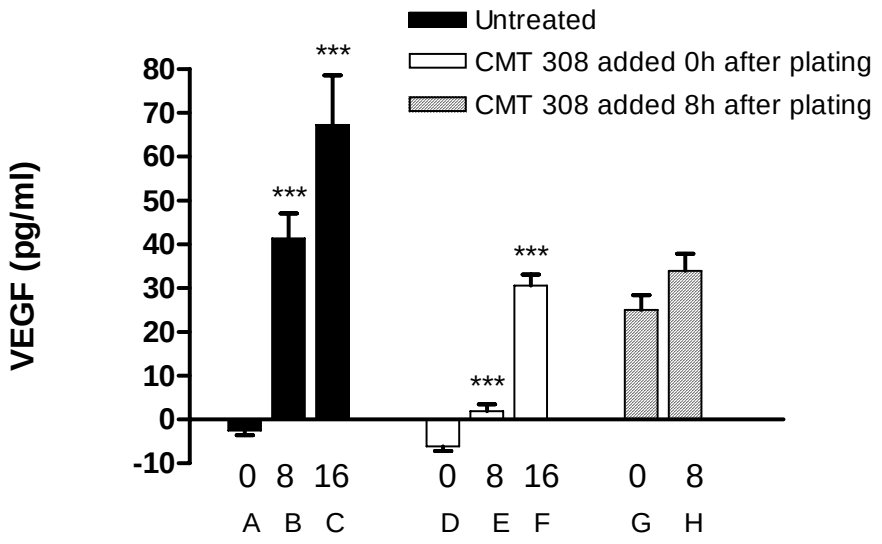

Untreated
CMT 308 added 0h after plating
CMT 308 added 8h after plating
VEGF (pg/ml)
80
70
60
50
40
30
20
10
0
-10
***
***
***
0 8 16 0 8 16 0 8
A B C D E F G H
Time after treatment with CMT 308 (Hours)

Fig 6

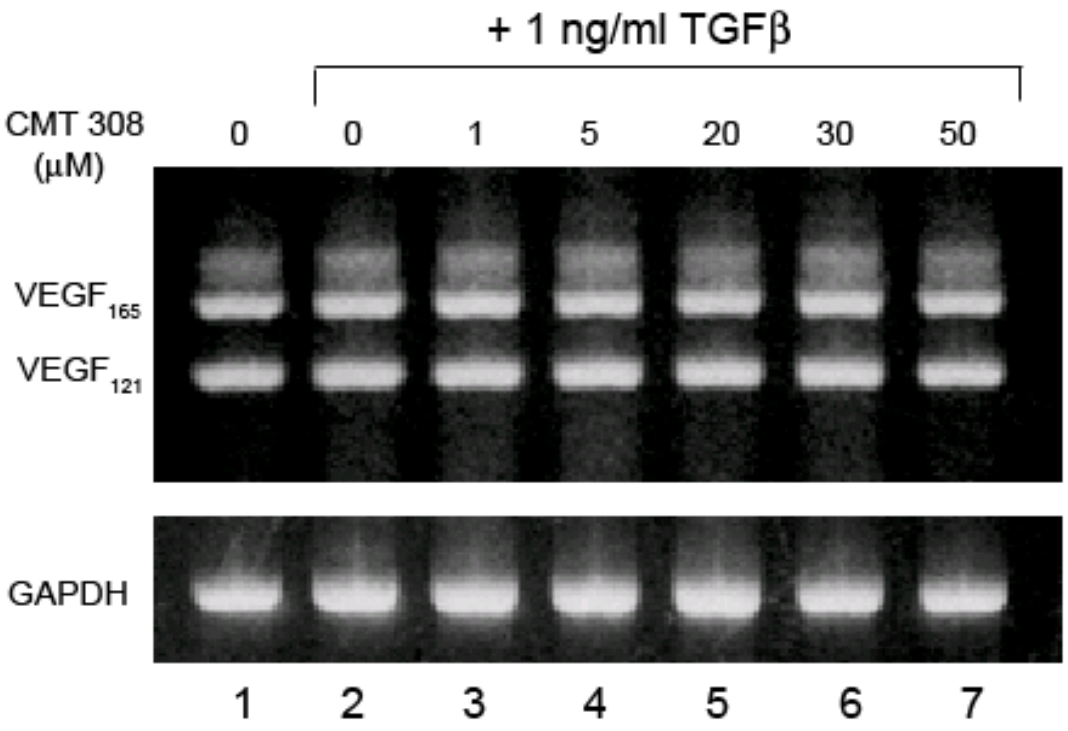

+ 1 ng/ml TGFβ
CMT 308
(μM)
0
0
1
5
20
30
50
VEGF165
VEGF121
GAPDH
1
2
3
4
5
6
7

**Fig 7A**

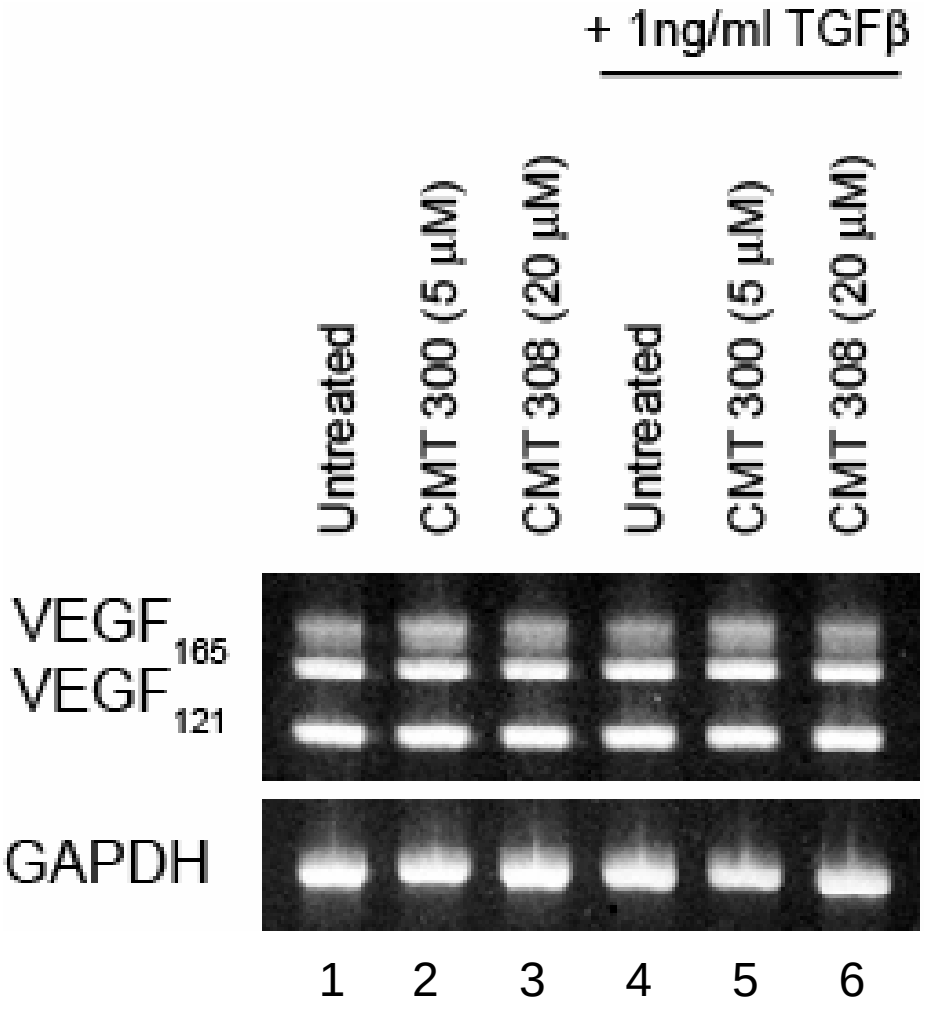

+ 1ng/ml TGFβ
Untreated
CMT 300 (5 μM)
CMT 308 (20 μM)
Untreated
CMT 300 (5 μM)
CMT 308 (20 μM)
VEGF165
VEGF121
GAPDH
1 2 3 4 5 6

**Fig 7B**

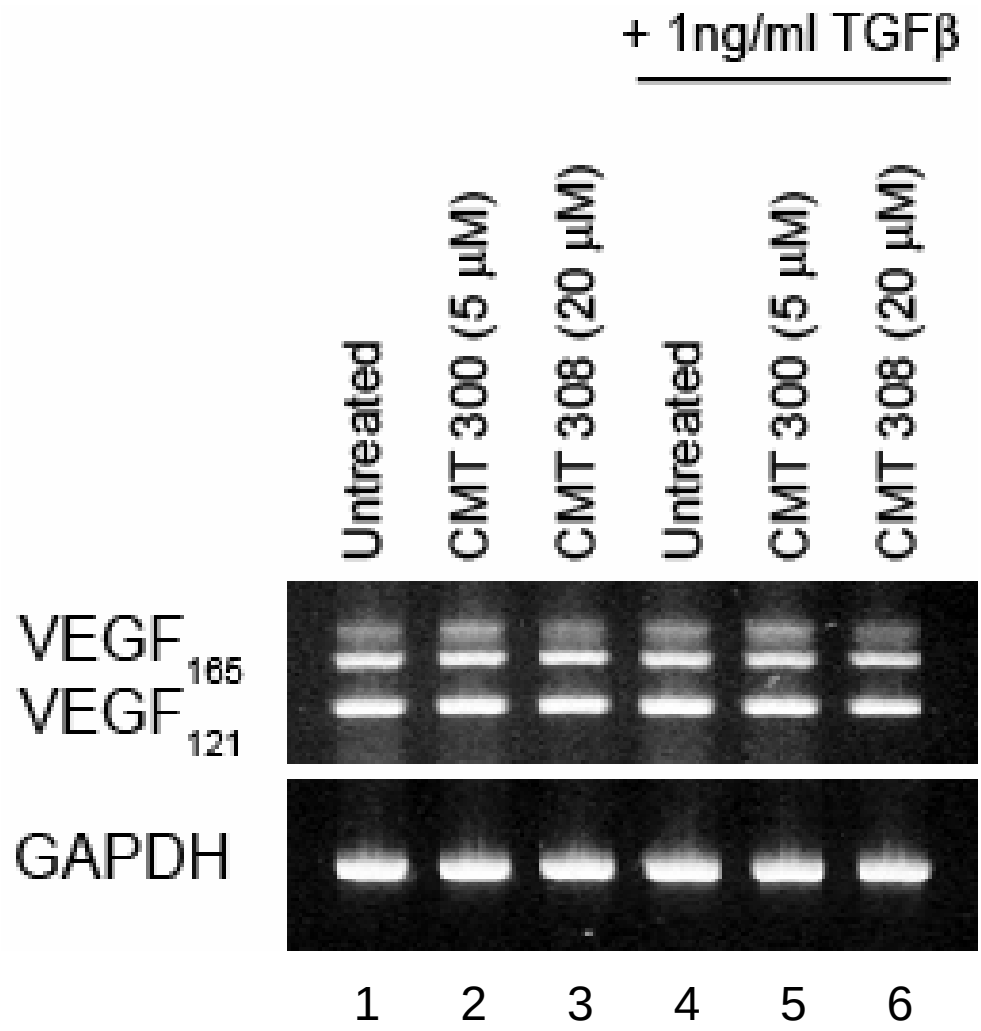

+ 1ng/ml TGFβ
Untreated
CMT 300 (5 μM)
CMT 308 (20 μM)
Untreated
CMT 300 (5 μM)
CMT 308 (20 μM)
VEGF165
VEGF121
GAPDH
1
2
3
4
5
6

**Fig 7C**

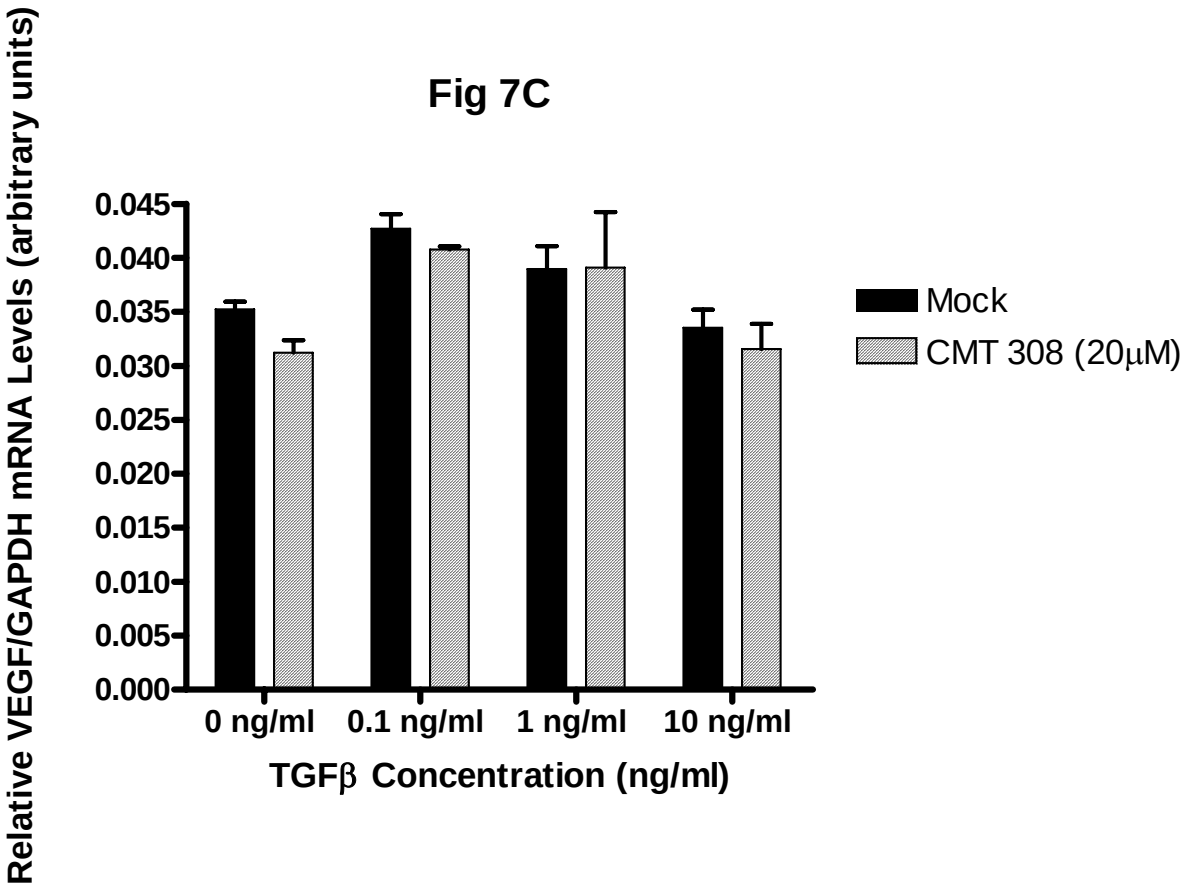
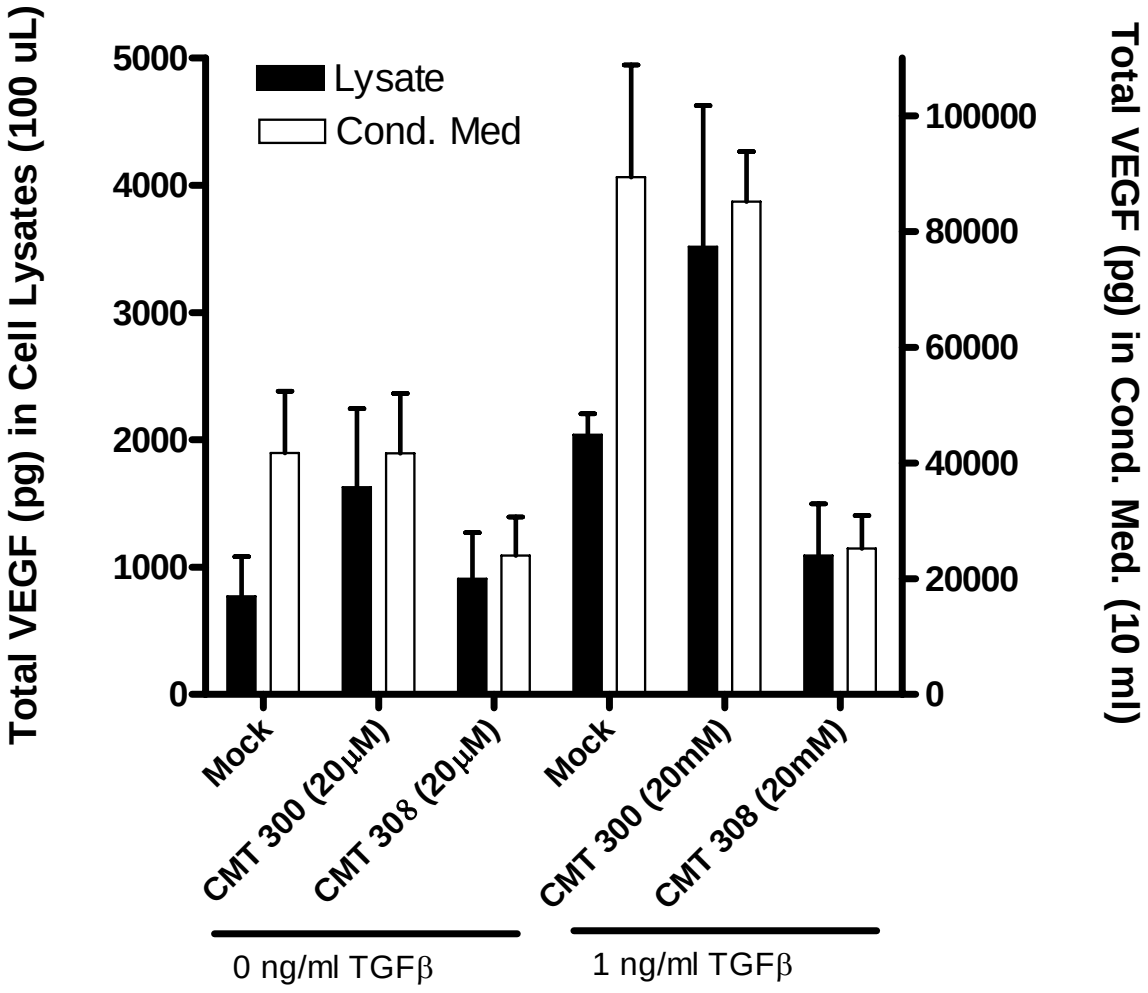

Relative VEGF/GAPDH mRNA Levels (arbitrary units)
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
TGFβ Concentration (ng/ml)
Mock
CMT 308 (20μM)

**Fig 8**


Total VEGF (pg) in Cell Lysates (100 uL)
Total VEGF (pg) in Cond. Med. (10 ml)
Lysate
Cond. Med
5000
4000
3000
2000
1000
0
100000
80000
60000
40000
20000
0
Mock
CMT 300 (20μM)
CMT 308 (20μM)
Mock
CMT 300 (20mM)
CMT 308 (20mM)
0 ng/ml TGFβ
1 ng/ml TGFβ

## Personnel

The following personnel have received support from this grant for their contributions to the research efforts summarized in this report:

A. Graduate Students (all students have since received the Ph.D. degree and have moved on to other positions) –

Mansi Kothari – The results summarized in this report formed the basis of Dr. Kothari's graduate thesis. She is first author on the two Era of Hope poster abstracts and the manuscript accepted for publication in the journal, *Cytokine,* all of which have been included in the Appendix.

Salih Kocer – Dr. Kocer's thesis research on the use of the chemically modified cytokines as metalloproteinase inhibitors was supported in its initial stages by this grant. He helped Dr. Kothari with visualization of VEGF isoforms by immnoblotting .His principal efforts on the use of CMTs as proteinase inhibitors were subsequently supported by a grant from NIH (NIAID).

Lori Seischab – Dr. Seischab's role on this project was primarily to assist Dr. Kothari in some of the techniques involved in visualization and quantitation of mRNA species for VEGF.

B. Senior Scientists (Research Associates) –

Elizabeth J. Roemer – Ms. Roemer was primarily responsible for the culture of all the breast tumor cell lines, the monocytoid line MonoMac 6, and the primary cultures of human microvascular and umbilical vein endothelial cells used in these studies. She also assisted Dr. Kothari in visualizing the formation of "tubes" and reticular networks formed by the endothelial cells.

QiLong Ying – Dr. Ying assisted Dr. Kothari in analysis of much of the data generated by her research using a number of numerical methods and graphical presentations.

C. Other Support Personnel

Wayne Bellucci – Dr. Bellucci assisted Dr. Kothari with experiments on breast tumor cell lines and MonoMac 6 cells while he was a medical student carrying out research in the laboratory.

Margaret Yazulla and Caroline Burns – Mss. Yazulla and Burns were responsible for administrative aspects of this project, including ordering supplies and maintaining appropriate records consistent with institutional policies.